Changes in behaviour and in protein and cholinergic markers in rat cerebral cortex during visual deprivation and controlled visual experience

Thesis

CHANGES IN BEHAVIOUR AND IN PROTEIN AND CHOLINERGIC MARKERS IN RAT CEREBRAL CORTEX DURING VISUAL DEPRIVATION AND CONTROLLED VISUAL EXPERIENCE

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ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
C	centigrade
CAT	cholineacetyltransferase
cm	centimetre
coA	coenzyme A
DF	degrees of freedom
DFP	diisopropyl fluorophosphate
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
DTNB	dithiobisnitrobenzoic acid
EDTA	ethylenediamintetraacetic acid
GAD	glutamic acid decarboxylase
h	hour
l	litre
M	molar
m	metre
mAChr	muscarinic acetylcholine receptor
mg	milligram
min	minute
ml	millilitre
nm	nanometre
PPO	1,2-diphenyldioxazole
QNB	quinuclidinylbenzilate
RNA	ribonucleic acid
RPM	revolutions per minute
RSA	relative specific activity
SA	specific activity
SEM	standard error of the mean
TCA	trichloroacetic acid
µl	microlitre
µm	micrometre

ABSTRACT

1. The sequelae of controlled periods of visual experience on the incorporation of precursor into protein and on the levels of enzymes involved in ACh metabolism were studied in the visual and motor cortex of Wistar rats following periods of visual deprivation from birth.

2. A small increase of incorporation of lysine into visual cortex protein in 7 week old dark reared rats was observed following a one hour period of visual experience, and a large increase was observed in both visual and motor cortex following visual experience immediately after normal eye opening.

3. Evidence was presented and discussed for the possible functional involvement of cholinergic synapses in the rat neocortex in an adaptive response to new visual experience.

4. The effects of three hour periods of exposure to light on visual cortex acetylcholinesterase activity were found to vary through the day, and a low amplitude rhythm of acetylcholinesterase activity was detected in the motor cortex of dark reared and normally reared animals. The behavioural activity cycle of dark maintained rats was found to vary diurnally with a rhythm which correlated with the motor cortex acetylcholinesterase activity.

5. Light induced elevations in visual cortex ACh enzymes and muscarinic receptor protein were present in rats of from one to two months of age following initial visual experience associated with mild and continuous activity in a motor driven apparatus, this procedure also resulting in some changes in motor cortex biochemical measures in dark reared animals.

6. The visual input during three hours of visual experience to 7 week old dark reared animals was controlled by the use of specially constructed corneal contacting lenses, and it was observed that the increases in visual cortex acetylcholinesterase activity were still present despite a substantial attenuation of pattern vision during light exposure.

7. The interpretation of these experiments is discussed in relation to relevant work in the literature in terms of the behavioural and biochemical significance for processes of neural plasticity.

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CHAPTER ONE

INTRODUCTION

It has become commonplace to acknowledge that the underlying neural mechanisms of behavioural plasticity remain an uncomfortably large grey area in our understanding of living systems. The contributions of neurochemistry in this area contend with a set of problems which are unique in the field of biology. This can be seen as a consequence of the entrepôt position of this field of enquiry, where questions asked in the horizontally structured field of biochemistry meet and integrate with the vertical structure of behavioural analysis and neurobiological theorizing. Added to this are the hurdles encountered by a relatively new and certainly rapidly developing science.

To proceed from the general to the particular: it is incomprehensible otherwise and hence axiomatic, that as a consequence of learning, involving some lasting change in an organism's behaviour, some neural changes occur within the organism. Theproblems then essentially become ones of first understanding the changes in the animals' behaviour - the focus of work here involving controls for behavioural processes which may or may not be essential to the processes of acquisition and recall (however demonstrated) and hence also storage, involving a temporal sequence, which are requirements of the learning process. Secondly, any neural correlates which are detected following the behavioural procedure must be demonstrated to be as specific as possible to these core processes. The neural correlates may be structural, physiological or biochemical, possibly all three, but often only one type is followed in any one experimental situation.

To take the behavioural considerations first.Defining

learning itself is no easy task. The concept has been widely, if not always correctly used to provide a unifying explanation of certain measurable changes in an animal's behaviour. Thus the distinction originally made between performance and learning by Hull (1943) is always worth bearing in mind. Whilst performance of an animal in a training situation may be described in a particular manner (dependent upon how measured), it cannot be assumed that learning underlying the behavioural change has so altered. An illustrative analogy may be drawn here from research on neural processes of motivation. Hunger as the hypothetical state of an organism will provide a unifying explanation for certain diverse patterns of behaviour, such as the amount and rate of ingestion of food, latency before eating, running speed in an alley to acquire food, the strength of current tolerated on an electrified grid to gain access to food, the number of unrewarded bar presses made in a Skinner box etc. These behavioural measures were correlated with responses in hypothalamic regions, the ventromedial nucleus (VMN) having been proposed (Anand and Brobeck, 1951) as a 'satiety centre' and the lateral hypothalamus as a 'feeding centre'. Further experiments to test this, involving lesioning the VMN, results in hyperphagic animals, with their 'satiety centre' obliterated. However, behaviourally these animals, although the quantity of food ingested is high in comparison to normal animals, appear less motivated to eat as scored by the amount of bitter tasting quinine tolerated in the food and degree of aversion to electric shock to perform the consummatory act (Teitelbaum, 1955). Valenstein et al. (1970) subsequently

demonstrated the particular behaviour elicited following hypothalamic stimulation was dependent upon environmental stimulus control factors, and proposed a learned component.

These studies indicate at least the importance of not relying on a single measure of behaviour. The pitfalls at the behavioural level of interpretation encountered in investigating neural correlates of learning - involving a particular experimentally induced altered behaviour pattern in an animal - are certainly no less. Fundamentally it can be asked, how applicable is imposing a task on an animal a general model for the processes which control learning? The active search loop in the learning chain, involving feedback of the $\overline{S \rightarrow R}$ type is often neglected in models involving passive 'exposure to' a stimulus situation. Neither can learning simply be regarded in general as a process of behavioural modification through experience, but must be regarded in the context of species-specific constraints on learning; not merely of the type accounted for in terms of differences in the sensitivity of peripheral receptors or in motor control, but centrally also. For example, the chaffinch can be made to learn the wrong pattern of song if recordings of adult call notes with the order of the phrases changed are played to it from birth, but it cannot be deceived into learning the songs of other species in this way (Hinde, 1970). Hence some perceptual filtering mechanisms subsuming templates can be regarded as fundamental. This may be linked to processes of attention and especially selective attention (Sutherland and Mackintosh, 1971) - what features of the stimuli present are analysed and discriminated and subsequently stored in the memory? Physiologically, this aspect may involve changes in efferent (centro-fugal) pathways to control incoming events.

Hence the animal is likely to be learning to attend to the relevant stimulus dimensions, and subsequently learns to attach the correct responses to stimuli having different values along these dimensions (generalization). Thereby learning involves a reduction of uncertainty of possible response outcomes in a particular situation, dependent upon the number of outcomes and the relative probability of occurrence of each alternative. Stimulus uncertainty is also important in relation to accuracy of recognition. Some of these issues can be illustrated by consideration of a model system of learning, the neurochemical correlates of which have been widely investigated (Horn et al., 1973; Rose, 1978b). This is imprinting in the chick, where the young bird learns to follow a natural or artificial stimulus. Here the sight of the imprinting object acts as a releaser of behaviour causing following - a response which has essentially been pre-programmed. What is being learned? - undoubtedly some specific perceptual features, and responses involving the elicitation of social behaviour (social attachment). An environmental stimulus thus acquires reinforcing properties (Campbell and Pickleman, 1961) during a particular developmental stage of the animal, whereupon the process of imprinting narrows the range of stimuli with these reinforcing properties.

In a training situation, the role of motivation is also likely to be important. Watts and Mark (1971) have used a one trial learning situation in the young chick where a single aversive experience (association of an evil-tasting substance with a coloured bead) subsequently substantially modifies the bird's behaviour towards that object (it refrains from pecking), and some biochemical correlates of this experience have been analysed. This can be interpreted as a disturbed motivation

to peck at a class of objects with which fear may be associated, or inhibition of a natural response to a class of stimuli, in addition to the learning of a specific visual discrimination.

Finally, the diversity of conclusions which behavioural analysts have produced following the study of different behaviour patterns subsumed under the learning mantle, is often more striking than the agreement. The focus of the neurochemistry of learning has in a sense spun off from the American behaviourist 'Skinner box technology' tradition, in which an adequate description of the learning phenomena has been assumed in terms of obtained conditioning performance curves of the standard laboratory rat. Situational determinants have been understressed. Thus the perception of stimulus relational elements which is essential to any learning task is partly under stimulus control, and interacts with species-specific constraints, maturational factors and with previous experience. The degree of plasticity is itself plastic. A few examples can illustrate these points. Pre-exposure to certain visual information during normal development has been shown to influence the subsequent learning of these discriminations in the rat (Oswalt, 1972) and in the chick (Chantrey, 1972). Deprivation studies have provided dramatic indications of the degree of plasticity (Held and Hein, 1963; Blakemore and Cooper, 1970) and provide an obvious area in which to study neural correlates. The process of learning can be envisaged as a complex interactive process between organism and environment, involving elements of feedforward from the stimulus and feedback from the results of actions. These are likely to involve changes in a number of neural systems. Attempts to extract and define common features of diverse learning phenomena must encompass observed behaviour as wide as unreinforced or

'incidental cue' learning of the rat in a maze, 'insight learning' in the chimpanzee first described by Kohler (1925) where a sudden gestalt results in a problem solved, and the cumulative formation of learning sets or 'learning to learn'. The overtraining reversal effect, with which the more a task is learnt - and hence presumably the more permanent the neural modifications - the easier it is for an animal to be trained to unlearn it, is also worthy of consideration.

These are some of the behavioural considerations. What of the approaches devised to encompass these considerations? It is not attempted here to review the voluminous literature on the neurochemistry of learning and experience which has now accumulated, but some specific experimental approaches are highlighted as illustrative. It is usually assumed that neurochemical changes detected or associated with one experimental learning situation - if adequate controls have been provided - will underlie other and different learning protocols, and that the specificity of the experience is encoded in other ways, possibly by the particular addresses of the cells involved. The durability of storage of a learned experience, after the process of consolidation into long term memory has occurred, is well known. Whereas short term memory processes require practice if they are not to decay rapidly, and are susceptible to physical and pharmacological disruption, a proportion of this information may be subsequently transferred to long term storage. It is assumed that structural changes occur in the connectivity of neurons involved. Most studies then have assumed the major site to be synaptic, although the way in which this process may occur is subject to considerable debate. A number of mechanisms are possible, and these may include the appearance of new synapses,

changes in the size of existing synapses, altered rates of production of transmitters and changes in the pre- and post-synaptic membranes.

There have been attempts also to identify unique changes in macromolecules to associate with a particular unique learned habit. Thus Ungar (1968, 1974) announced the isolation of a peptide which was claimed to mediate dark avoidance in rodents, a habit which it was also claimed could be transferred to naive animals by injection of this substance. It has also been proposed that some of the endogenous peptides and peptides formed in nervous tissues may be putative transmitters (Reichelt and Edminson, 1977; Iversen, 1978) and that these may mediate memory (De Wied, 1977; Reichelt et al., 1978). There now seems little doubt at least that the spate of dramatic early experiments reporting the successful interanimal transfer of learned habits in planaria (McConnel, 1966) or vertebrates (Babich et al., 1965) mediated through the injection of trained animal homogenate, brain homogenate, specific RNA enriched extracts, or proteins, can all be dismissed on a variety of sound theoretical grounds (Rose and Longstaff, 1979) and from a subsequent history of failures of independent replication of these results.

Another approach has involved the administration of specific inhibitors or facilitators of various biochemical mechanisms, and examined the time course of their effects on the acquisition and recall of learned responses. Thus Deutsch (1971) using the intracerebral injection of diisopropyl fluorophosphate (DFP), an anticholinesterase, has trained animals on a Y maze and reported facilitation of this habit if animals were injected 28 days later after forgetting had occurred, but blockage of the habit if injection occurred shortly after training. The effects of

injection of an anticholinergic were opposite. From these and similar experiments, it was concluded that as a result of learning, the postsynaptic endings at a specific set of synapses become more sensitive to the transmitter.

A number of groups have employed inhibitors of RNA and protein synthesis. Actinomycin D, a blocker of RNA synthesis, has been reported also to block memory after in vivo injection in the goldfish and rodent (Agranoff et al., 1965; Squire and Barondes, 1970). On the other hand, blockers of DNA synthesis such as arabinosyl cytosine have shown no effect on learning and memory where they have been used in a similar situation (Casola et al., 1968), and concepts of cell regulation do not normally imply that DNA turns over. Thus a block at the transcriptional level has been reported to produce similar effects on memory storage to the large body of experimental evidence implicating protein synthetic mechanisms. However, the interpretation of these experiments is not at all obvious; the results are partly species-dependent, the agents themselves are extremely toxic and have a diversity of behavioural and biochemical sequelae, and it would perhaps be surprising if some effects on learning in some situations had not been demonstrated.

The agents used to block protein synthesis have generally less toxic effects, and a great number of experiments have been reported with their use. In vivo administration has been demonstrated as disrupting the retention of learned responses in the goldfish (Agranoff et al., 1965) and the rodent (Flexner and Flexner, 1965) which was dependent upon the time of injection after training. Flood et al. (1975) have subsequently demonstrated that the injection of anisomycin at various times after training results in a progressive loss of retention, dependent upon the extent of the protein synthesis blocked.

Some of these agents have been shown to have effects other than on protein synthesis - puromycin, for example, has been shown to cause mitochondrial swelling, and cycloheximide to lead to lowered noradrenalin levels (Flexner and Goodman, 1975). Behaviourally, some of the agents involved have also been shown to have other effects than on memory; the pattern of overall motor activity in rats, for example, is altered by cycloheximide (Squire and Barondes, 1970) and anisomycin (S.P.R. Rose and N. Wood, unpublished), although these effects have been claimed to be independent of the effect on retention (Segal et al., 1971).

Although individual experiments can be criticised, the overall evidence from inhibitor studies would indicate that protein synthesis is a requirement for the retention of long-term memory. Further, the use of more specific inhibitors of cellular mechanisms may lead to an increased understanding of the time course of the events involved in consolidation. Thus, largely on the basis of the time course of the effects of several different inhibitors, McGaugh (1968) and Gibbs and Ng (1977) have proposed three phase models of memory consolidation, although these must remain no more than speculative working hypotheses.

Finally, a number of studies have sought to detect regionally specific changes primarily involving RNA, proteins or enzymes following a learning experience in the absence of blocking agents. While in principle this approach is probably capable of providing the most direct evidence of the involvement of these molecules, the practical difficulties of behavioural control and the problems of biochemical interpretation are large. The earliest and probably best known experiments are

those of Hyden et al. who, using microtechniques of hand dissection of neurones and glia,reported (Hyden and Egyhazi, 1962) changes in total RNA and the base ratios of isolated RNA of cells in Dieters nucleus in animals which had learned to balance on an inclined wire, but which were not present in passively stimulated controls. The behavioural control for vestibular stimulation was, however, not readily comparable. An improvement of controls was achieved by training rats to reach for food with a non-preferred paw, using each animal as its own control. Similar changes in RNA to those he had reported earlier were observed (Hyden and Egyhazi, 1964) in addition to an altered pattern of ^{3}H leucine incorporation into proteins (Hyden, 1973) of the sensori-motor cortex, and increases in the brain specific protein S-100 and of calcium ions in a hippocampal region (Hyden and Lange, 1970) - an antibody to which was reported to prevent learning of the skill (Hyden, 1973). These experiments represent a considerable body of evidence in respect of the particular changes detected following a learning task, and although subject to both behavioural and biochemical difficulties (Rose and Haywood, 1977) concerning their unequivocal interpretation, a number of other studies, using a variety of training methods, have similarly reported comparable changes in RNA and protein synthesis in several species, although inevitably inconsistencies arise.

Hershkowitz et al. (1975) have reported a pattern of changes in different amino acids incorporated into brain protein following learning in the rodent of a positively reinforced task, Popov et al. (1976) the enhancement of labelled fucose incorporation following learning in the rat

of a visual task (a shock motivated brightness discrimination), and Shashoua (1970, 1976) the enhancement of incorporation of precursors into RNA and into specific protein fractions in the goldfish brain following an adjustment to swimming in an unstable state (although the control for differential stress effects would not appear to be complete) - which proteins tended to be secreted into CSF during training and antibodies to which tended to impair the retention of the behaviour (Shashoua and Moore, 1978).

None of these experiments, nor many others not mentioned here, have conclusively individually demonstrated the involvement of these macromolecules specifically with learning to the exclusion of an alternative explanation, and this reflects the difficulties inherent in devising sufficiently adequate controls, for example, for aspects of differential stress, motor activity, handling effects, motivational factors and attention (Bateson, 1976) - some of which have been shown to influence incorporation rate in ostensibly 'non-learning' situations (Rees et al., 1974; Jakoubek et al., 1970). Alteration in precursor incorporation could reflect changes in the labelled or unlabelled precursor pool sizes, and degradation as well as synthesis of macromolecules may be altered during experimental treatment. Even where controls or methods of calculation have been employed to minimise these possibilities, alterations in protein synthesis itself may be only a general correlate of the experience and need not be central, or it may indeed be only part of a much larger metabolic mobilization.

One of the most sophisticated long-term attempts to delineate further both the biochemical and behavioural aspects of a learning experience has been the imprinting experiments in the young chick (Horn et al., 1973; Rose, 1976b) referred to earlier. An ordered temporal sequence of events following this experience have been described; these include transient fluctuations in several

enzyme levels, including the enzymes of ACh metabolism and of RNA synthesis, enhanced incorporation of uracil into RNA and of amino acids into proteins, especially in one particular forebrain area. Significant positive correlations between one particular measure of habit strength and the amount of incorporation of uracil into RNA and of leucine into a tubulin rich protein fraction in this one brain area have been detected. Furthermore, a variety of behavioural controls have rendered it improbable that one of these findings - the increased incorporation of uracil into RNA - is an effect associated with stress, sensory stimulation per se, or altered motor activity.

All the experiments so far described have attempted to relate neurochemical changes directly to a training experience; another type of approach has concentrated on the neural correlates of novel experience in non-training situations, and has been primarily concerned with transient and long-term aspects of neuronal plasticity. This has involved subjecting animals to some profound changes in environment during development. This approach possesses the particular advantage that morphological and physiological studies can complement the biochemical findings. The experimental procedure generally involves rearing animals under varying degrees of restricted experience - often, for ease of practical control, involving the visual modality - sometimes followed by controlled periods of altered experience. By this means it is possible to explore the nature and extent of behavioural and neural plasticity. The adaptive changes which occur under these conditions have, in general, been assumed to be broader and larger scale analogues of the changes which may occur and underlie adaptation to a particular learning experience (Rose, 1977, 1978).

In the rodent, morphological studies (Valverde, 1967) indicate a reduction of the number of spines on the apical dendrites of pyramidal cells in the visual cortex occurring as a result of 20 days of dark rearing in the mouse, and which is followed by the growth of new spines after visual experience (Valverde, 1971). Changes in the number and density of synapses have also been reported (Cragg, 1967, 1969) as a consequence of visual experience following 50 days of dark rearing in the rat in both the visual cortex and lateral geniculate nucleus. Borges and Berry (1978), using Golgi-Cox methods,have demonstrated a pattern of reorganisation of dendrites, and changes in the glial/neuronal cell ratio in layer IV of the visual cortex of dark reared and subsequently light exposed rats compared to normal animals.

Studies on the manipulation of visual experience in the cat have particularly provided examples of dramatic parallel morphological, physiological, biochemical and behavioural alterations. As an example, Spencer and Coleman (1974) have observed changes in the dendrites of stellate cells in layer IV and of pyramidal cells of layers III and IV of the visual cortex which are influenced by the direction of stripes experienced during dark rearing, while Blakemore and Cooper (1970) reported significant modification of the receptive field properties of visual cortex neurones in animals reared during a critical period with visual experience only of stripes in one direction, and which was reflected by appropriate behavioural deficits in visual performance. Binocular deprivation of visual input from birth has been shown to result in increased incorporation of ^{14}C leucine into visual cortex proteins following unilateral visual stimulation (Skangiel-Kramska et al. 1978)paralleled by a number of

physiological (Blakemore, 1977) and morphological (Garey and Pettigrew, 1974) cellular changes.

Regionally specific biochemical changes following dark rearing and initial visual experience have been reported in a number of species. Increased incorporation of amino acids into visual cortex proteins has been found in the monkey and rabbit, following exposure to flashing light (Talwar et al., 1966; Singh and Talwar, 1969) which was a function of the light intensity. Murthy et al. (1977) have reported decreases in visual cortex cytoplasmic RNA and membrane bound polysomal changes in four week old dark reared rats, and an increased incorporation of precursors into proteins and RNA following exposure to flashing and continuous light of varying wavelengths. Tubulin synthesis has been reported to be depressed in the visual cortex of dark reared rats and elevated following eye opening and visual experience (Perry and Cronly-Dillon, 1978). Mareš et al. (1975) have reported a significant developmental alteration in the rate of glial proliferation in the lateral geniculate nucleus and visual cortex of deprived rats, and changes in the levels of several transmitter related enzymes in the visual cortex, lateral geniculate nucleus and superior colliculus of dark reared rats have been found (Maletta and Timiras, 1967, 1968; Bigl et al., 1974a, 1974b; Meisami, 1975).

On ths basis of these and similar observations, it has been proposed that sensory input partly shapes the process of synaptic development in the visual system of the mammal, although undoubtedly the sensitivity and time dependency of the effects vary greatly amongst different species. This approach should be capable of distinguishing which plastic neural

adaptations to experience are transient and reversible, are longer term, and are interacting with normal neural developmental processes. In this sense all of the approaches which have been described complement each other. As a framework of interpretation, it has been proposed (Rose, 1977, 1978a) that three general classes of neurones exist in the brain; those which have connections specified independently of experience, those which are modifiable only during a sensitive period in development, and another category in which synaptic modulation may occur in response to novel experience throughout life.

This general paradigm is adopted in the experiments described in this thesis. The experimental situation employed in this project is the first exposure of dark reared rats to the light. This model system, originally exploited by Rose (1967), has since been the focus of considerable biochemical investigation and a coherent pattern of changes has been detected. The merits may be summarized. The laboratory rat has been the subject of extensive basic biochemical and behavioural research, and hence ready comparison of results is possible. The visual system is well understood at the anatomical and physiological levels. Experimentally, alterations in visual input can be readily and easily controlled in comparison to the other sensory modalities. Although visually mediated behaviour may not appear to be the obvious choice of study in the albino rat, it is known that extensive periods of dark rearing produce no severe pathological changes in the visual system of the type known to occur in some other mammals (Reisen, 1950). Dark reared rats show only some minor decrements in visual ability during initial visual experience, when compared with the normal abilities of animals reared throughout their lives in a visual

environment. The particular design employed in these experiments enables comparisons to be made between dark reared animals and normally experienced litter mates, in addition to animals which have received short periods of visual experience at any time after birth.

As has been briefly described, a number of comparable studies on dark reared rodents have demonstrated measurable morphological changes in the visual system at the E.M. and light microscopic level as a result of the altered experience, and direct parallel microanatomical work using the same experimental design has been performed (Cragg, 1967, 1969), in addition to other comparable biochemical studies. Previous work from the Open University Brain Research Group has shown that the rate of incorporation of precursors into proteins of the visual system of 50 day old dark reared rats is less than that of normal animals, and is transiently increased following one to three hours of visual experience (Richardson and Rose, 1972; Richardson, 1974). These changes have been detected at the cortical, geniculate and retinal levels, and the increased incorporation is only into a limited number of proteins (Richardson and Rose, 1973a, 1973b). After one hour of light exposure, much of the elevation in the visual cortex is ribosomally bound (Jones-Lecointe et al., 1976). The cellular locus of these effects indicates the elevation is confined to a neuronal fraction, and there is an increase in the rate of incorporation into a rapidly labelling and exported neuronal glycoprotein fraction (Rose and Sinha, 1974), switched on within an hour of visual experience into a tubulin rich fraction (Rose et al., 1976). This latter is a lasting effect, in contrast to a transient response to visual experience. Other lasting changes to visual experience include an increase in the levels of several lysosomal enzymes (Sinha and Rose, 1976). More transient visual

cortex modulations to experience, in that there is an increase in light exposed animals over both normal and dark maintained animals which is detectable after several hours but not 24 hours of exposure, include the synthesis of particulate glycoproteins (Burgoyne and Rose, 1978), and an increase in a tubulin rich fraction (Stewart and Rose, 1978). A further class of short term changes involves the enzymes of ACh metabolism (Sinha and Rose, 1976) and the muscarinic cholinergic receptor (Rose and Stewart, 1978).

The present project has concentrated on the changes in cholinergic components, and in particular the degradative enzyme acetylcholinesterase, with which to investigate some stimulus control procedures. The accumulating evidence of the specific biochemical alterations associated with visual experience posed some specific questions concerning the relevant features of the total environmental stimulus, and the behavioural context in which they occurred. It was not practicable within the time constraints of the present project to investigate more than one or two biochemical markers in which to control behavioural procedures, and the emphasis here has been to provide a further description of some of the variables involved with the acetylcholine system. With these constraints in mind, the aims of this project were conceived in general terms as below:

(1) To relate biochemical modulations in the cerebral cortex to specific aspects of altered experience, to determine the extent and nature of the state-dependent correlations.

(2) To measure aspects of altered behaviour produced during dark rearing and initial visual experience, and to attempt to correlate these with the biochemical measures.

(3) To design and employ experimental treatments to control the non-visual variables and the nature of the visual experience during first exposure.

(4) To determine if the biochemical responses are to some extent dependent upon the length of dark rearing, and to relate these to ongoing processes of maturation and development of the nervous system.

(5) To interpret the significance of the biochemical responses with respect to the function of the state dependence in terms of the results, and of findings from other sources.

Within these general considerations, Chapter 2 presents results obtained using the incorporation of labelled lysine into visual cortex proteins under different exposure regimes, using immature and young adult animals, and provides some preliminary but suggestive results as an introduction to this project. Chapter 3 provides a more detailed survey of the cholinergic system and its relationship to plasticity in behaviour, and Chapter 4 describes the practical biochemical assay procedures employed in this study. Chapter 5 presents the results of experiments indicating the influence of circadian fluctuations in levels of enzyme, in the biochemical response to visual experience, and in behaviour during visual experience. One aspect of behaviour during visual experience -overall motor activity - and its relationship to cortical changes in ACh enzymes and receptor protein was further investigated in animals of different age groups. These results are presented and discussed in Chapter 6. Chapter 7 describes the results of experiments designed to define more precisely the relevant features of the visual experience during light exposure. Finally, Chapter 8 provides a synthesis of the main conclusions of this research, together with their possible significance, and indicates some further possible directions for future work.

CHAPTER TWO

INCORPORATION STUDIES - REPLICATION AND EXTENSION

Experiments were designed initially to replicate the elevated incorporation of ^{3}H lysine into acid precipitable proteins which has been shown to occur during a one hour exposure to light, using the 50 day old dark reared rat, and to subsequently extend these procedures to animals of different ages. Firstly, some preliminary work was required. In particular, it was necessary to establish the temporal parameters of incorporation of the precursor into visual and motor cortex proteins as determined in the homogenates. Although this work has been described by Tiplady (1972b) and Richardson (1974), it was deemed essential to ensure that the optimal incorporation period had remained similar, due to the possibility of genetic drift or other factors having occurred in our closely inbred colony of Wistar CFHB animals.

Materials and Methods

Animals

The animals employed in these experiments were all male Wistar rats from the CFHB strain born and reared in the animal house at the Open University Department of Biology, and used when 50±5 (range) days of age, at which time they weighed 140-200 grams in these experiments. These animals were reared under approximately a 12 h light/12 h dark lighting regime, kept in cages 57 cm x 39 cm x 18 cm, and allowed food and water *ad libitum*.

Procedure

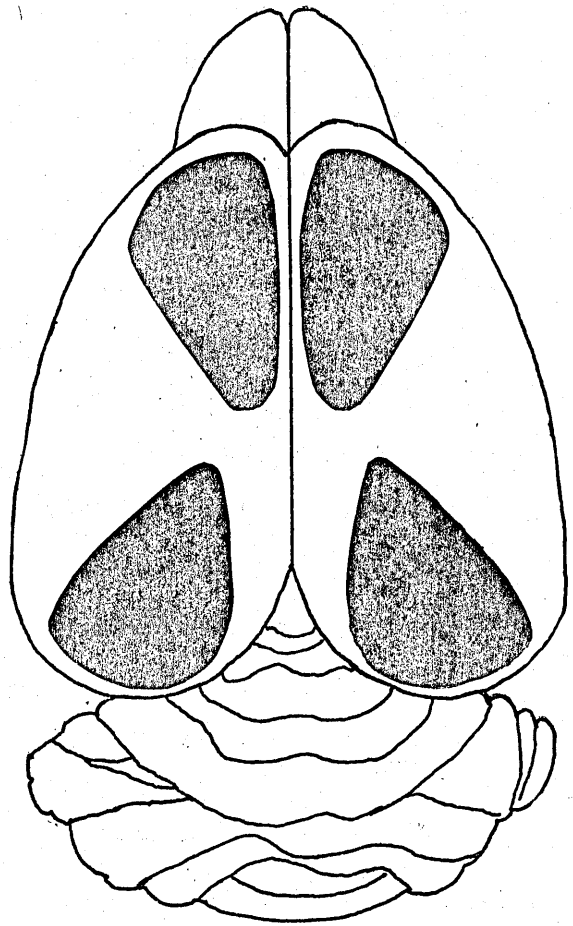
L-(4,5(n)-^{3}H) lysine monohydrochloride (Radiochemical Centre, Amersham, Bucks.) was used as the chosen precursor in these experiments, having been shown (Lajtha and Marks, 1971) to have a

suitably high facility of uptake into the precursor pools in the brain. Although isotopic exchange of tritium with water seems to occur within an hour of administration (Tiplady, 1972b), it has not been shown, nor does it seem reasonable to assume, that the rate of isotopic dissociation is significantly altered by one experimental condition rather than another, (although the rate of uptake may vary). Against this is balanced the greater number of animals which can be used at similar cost compared with using the carbon labelled compounds. All animals were injected intraperitoneally with 50 µCi in 0.5 ml of 0.9% saline, this route having been shown (Tiplady, 1972b) to result in good reproducibility and to cause a minimum of stress.

Immediately after injection, animals were placed in individual cages in the open laboratory for the following time periods: 15 mins, 30 mins, 60 mins, 120 mins. After each time period, three animals were killed by a blow to the back of the neck, and the brains rapidly removed (< 45 secs) and placed on Petri dishes cooled on ice.

Visual and motor cortices were dissected and cleaned of underlying white matter. In addition to the primary visual projection area (striate cortex) some visual association areas were taken as visual cortex, making the total cortical visual area removed some 80-100 mg (wet weight). A similar quantity of cortical matter was removed from the anterior portion of the brain comprising frontal (motor) cortex. Fig. 2.1 illustrates the location and extent of the cortical areas removed. Left and right halves of each brain were pooled and homogenized in 5.0 ml of 0.9% saline using an MSE top drive macerator at 14000 RPM for 60 secs. Three aliquots (each in duplicate) of

Fig. 2.1



Regions of cortex sampled.
Posterior dark areas =
visual (striate + association)
cortex.
Anterior dark areas =
motor (frontal) cortex.

each of these homogenate samples were taken and processed as follows:

(1) 1.0 ml was taken and precipitated for at least one hour with ice-cold trichloracetic acid (TCA) containing 10 mM lysine to displace free radioactive precursor. These were centrifuged at 2000 RPM for 15 mins and the supernatant discarded. The pellets were fragmented and washed three times by resuspending in TCA followed by further centrifugation. The pellets were dried, then solubilized in 0.3 ml of NCS (Amersham/ Searle, High Wycombe, Bucks.) at 50° C for 1-3 h. The tubes were washed out with 10 ml scintillant containing 6 g/l,2-diphenyldioxazole (PPO) and toluene and methoxyethanol in equal proportions. These were counted on a Beckman LS 150 scintillation counter, and quench corrected by the external standard method according to standard quench curves.

(2) A second aliquot of 0.5 ml was taken and immediately solubilized and subsequently treated as described under (1) above, for estimation of total radioactivity in the homogenate.

(3) A third portion of 100 µl was taken for protein estimation by the method of Lowry et al. (1951) in a total volume of 4.5 ml and read against a distilled water blank using a BSA standard (1 mg/ml) on a Bausch and Lomb Spectronic 700 spectrophotometer.

The incorporation of the precursor was expressed in two ways - specific activity (SA) which was defined as disintegrations/ minute/mg protein in the acid precipitable material, and relative specific activity (RSA) obtained by calculating the ratio of disintegrations/minute in the TCA insoluble material to that in the total homogenate, and multiplying this by 100 to express it as a percentage.

The results of these time course experiments are shown graphically in Figs. 2.2 and 2.3, which represent the means ± S.E.M.. Acid insoluble radioactivity increased steadily up to one hour after injection, very similarly in both brain regions, and there was no appreciable further increase during the subsequent hour. This was evident in both the SA and RSA measures. These results are very similar to those reported by Tiplady (1972b) and Richardson (1974). The standard errors on the RSA measures are smaller than those for the SA figures, reflecting the greater reliability of this measure (Rose, 1967). Having confirmed the suitability of this experimental control procedure, a one hour exposure period with a simultaneous pulse of radioactivity was chosen for subsequent experiments involving the exposure of the 50 day old dark reared animals to the light.

These initial experiments were directed at replicating the regional specific elevation of incorporation of the ^{3}H lysine into proteins as determined in unfractionated homogenates by Rose (1967) and Richardson and Rose (1972), which is consequent upon exposing the 7 week old visually inexperienced rat to a one hour period of visual experience.

The biochemical procedures are essentially identical to those reported above for the time course experiments. The difference in the animal rearing procedure is as follows. Litters were reared by placing the pregnant females prior to parturition into cages measuring 36 cm x 24 cm x 10 cm and disposing these inside light-tight wooden boxes which were stored in adjacent positions on racks in the animal house. Feeding and watering of animals and cleaning of cages was carried out twice weekly, as quickly as feasible, under illumination from a dim red lamp - a spectral frequency range to which the albino rat is almost

Legend to Figures 2.2 and 2.3

Time course of incorporation of ^{3}H lysine into TCA insoluble material in visual (broken line) and motor (solid line) cortex. 50 day old normally reared rats were injected intraperitoneally with 50 µCi ^{3}H lysine and incorporation determined at the times shown. Fig. 2.2 represents RSA's (TCA insoluble DPM/total homogenate DPM X 100%) and Fig. 2.3 SA's (TCA insoluble DPM/mg protein). Each point represents the mean ± S.E.M. of 3 determinations.

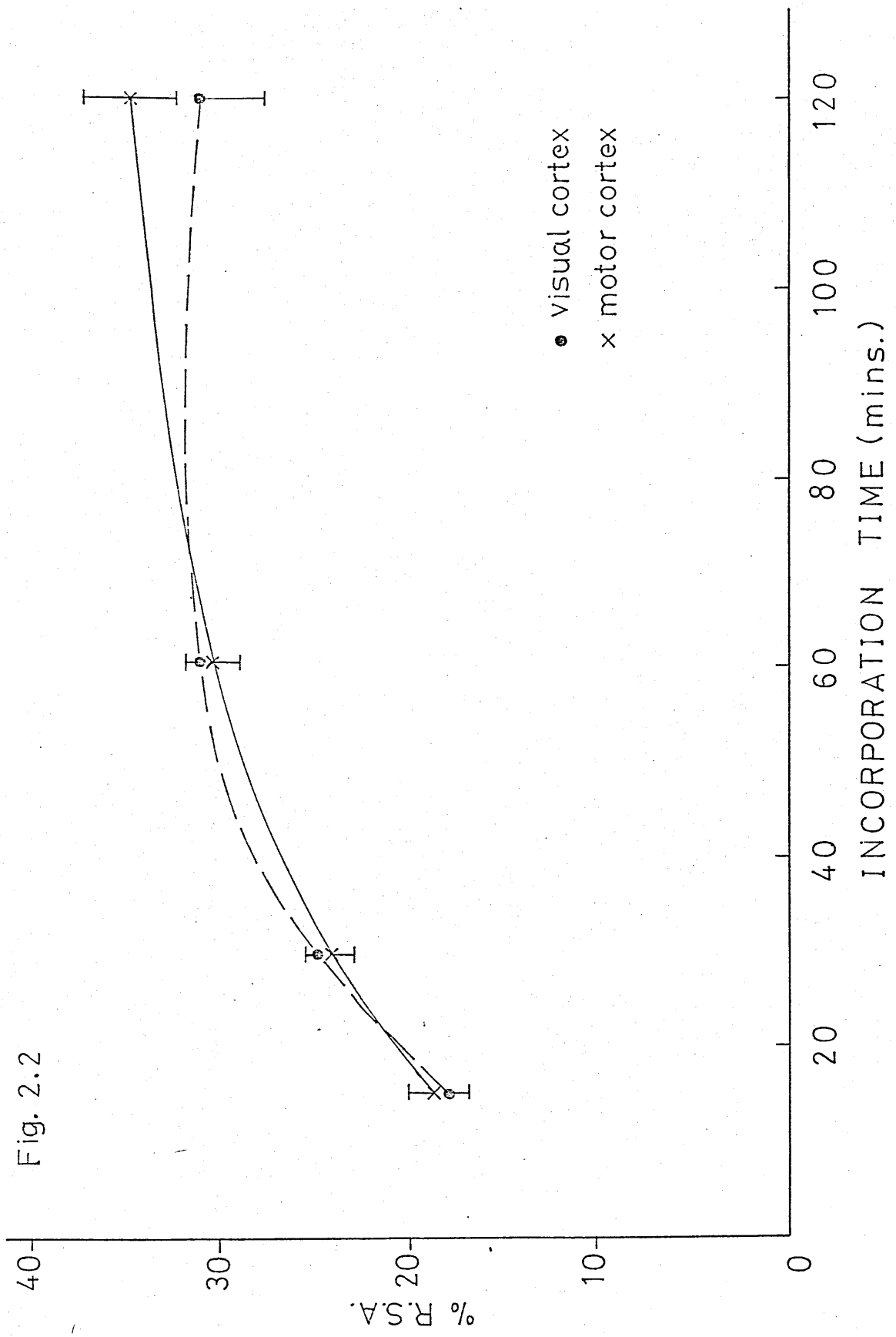
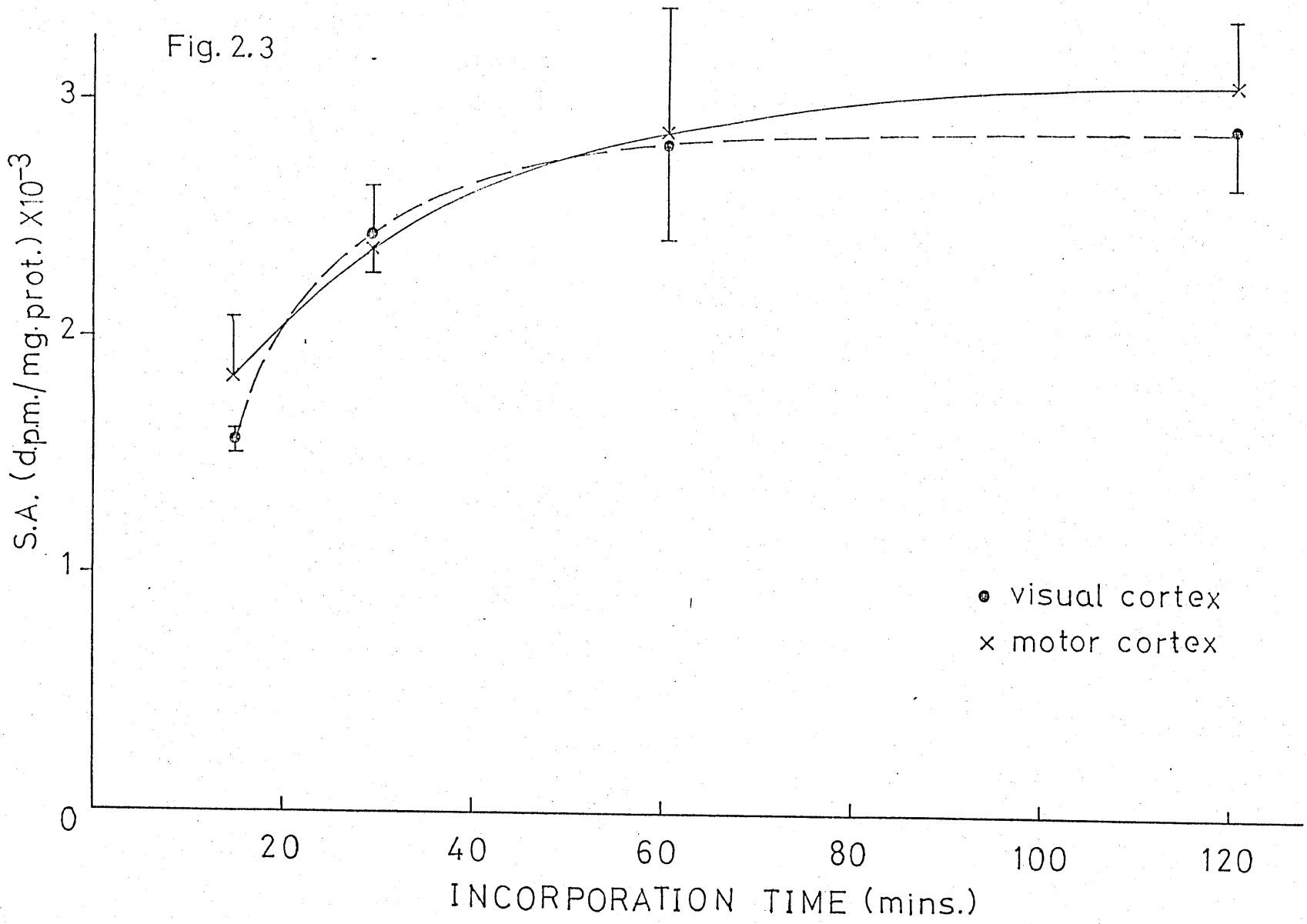

40
30
20
10
0
% R.S.A.
20
40
60
80
100
120
INCORPORATION TIME (mins.)
• visual cortex
× motor cortex

Fig. 2.2

Fig. 2.3


S.A. (d.p.m./mg.prot.) X10⁻³
0
1
2
3
20
40
60
80
100
120
INCORPORATION TIME (mins.)
visual cortex
motor cortex

insensitive (Silver, 1967). At 21-24 days after birth of the litter, the mother, the female infants and, in large litters, approximately one third of the male animals were removed. The removed male infants were transferred to a single cage in a different part of the same animal house. These animals were kept under conditions of approximately 12 h light/12 h dark, and used as normal controls (Normals, N) at 50 ± 5 (range) days of age at which age they weighed 180 ± 40 (range) grams, a range not significantly different from their dark reared littermates, or from animals reared under normal animal house conditions for their entire lives.

Animals were exposed to illumination (luminous flux 1000 to 5000 lumens/m^2) by transferring the dark rearing boxes to an open laboratory and randomly selecting about half of the dark reared animals to be exposed in adjacent individual cages on a bench. The dark reared and unexposed animals were individually transferred to fresh dark boxes on the same bench after injection under a dim red lamp. Plate 1 illustrates the conditions of a litter of animals inside an opened dark box, and Plate 2 illustrates the typical experimental conditions of the light exposed animals during the exposure period.

As previously defined, the brain regions examined were visual and motor cortices in each case. Left and right halves of each brain region were routinely processed separately, and the results arithmetically summed and averaged at the end. The means ± S.E.M. of the results of these experiments are illustrated in Figs. 2.4 and 2.5. These results represent data standardized in the following manner. All specific activity results were normalised around a standard body weight of 150 g (i.e. SA = X, body weight

Plate 1. Rearing conditions inside a dark box

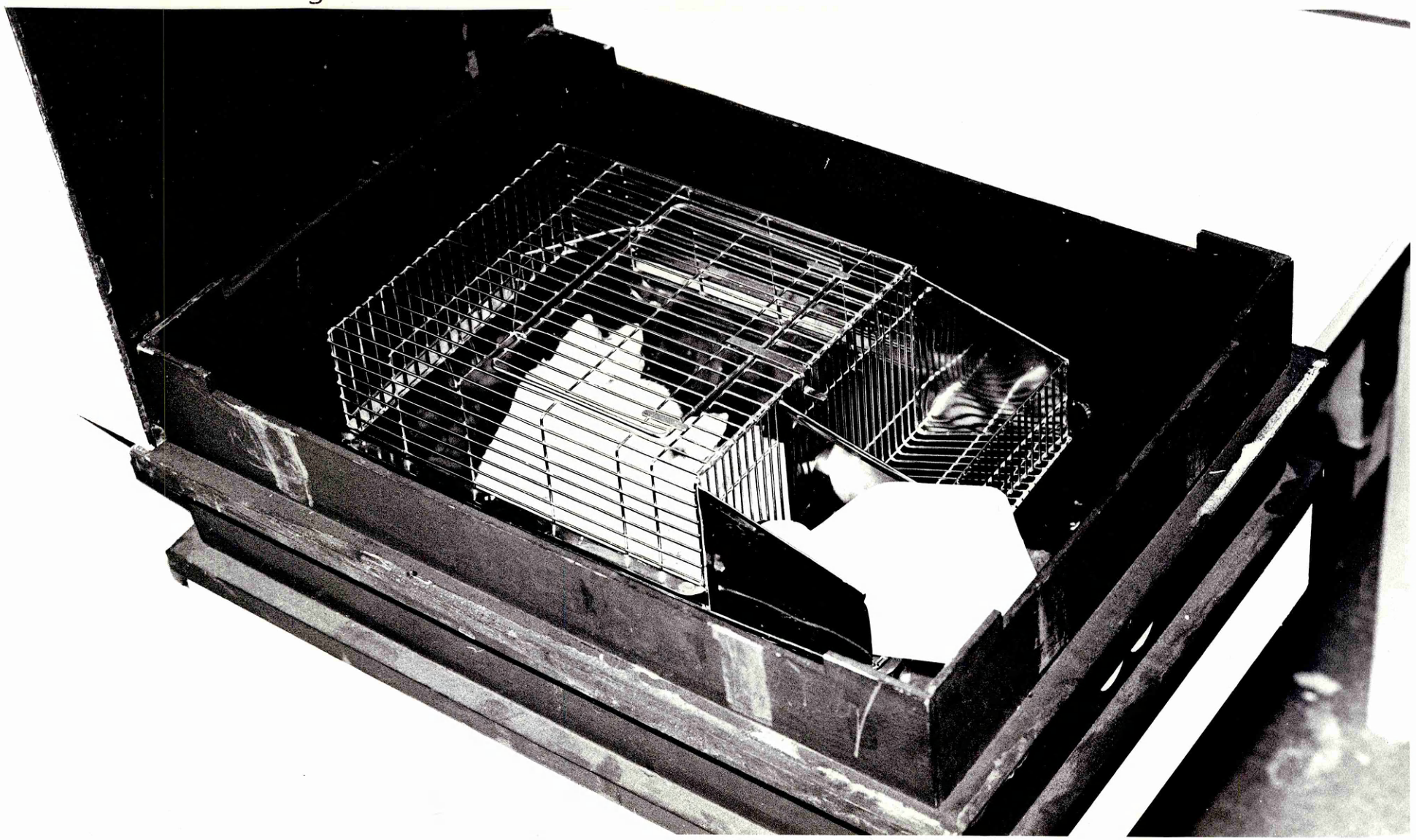
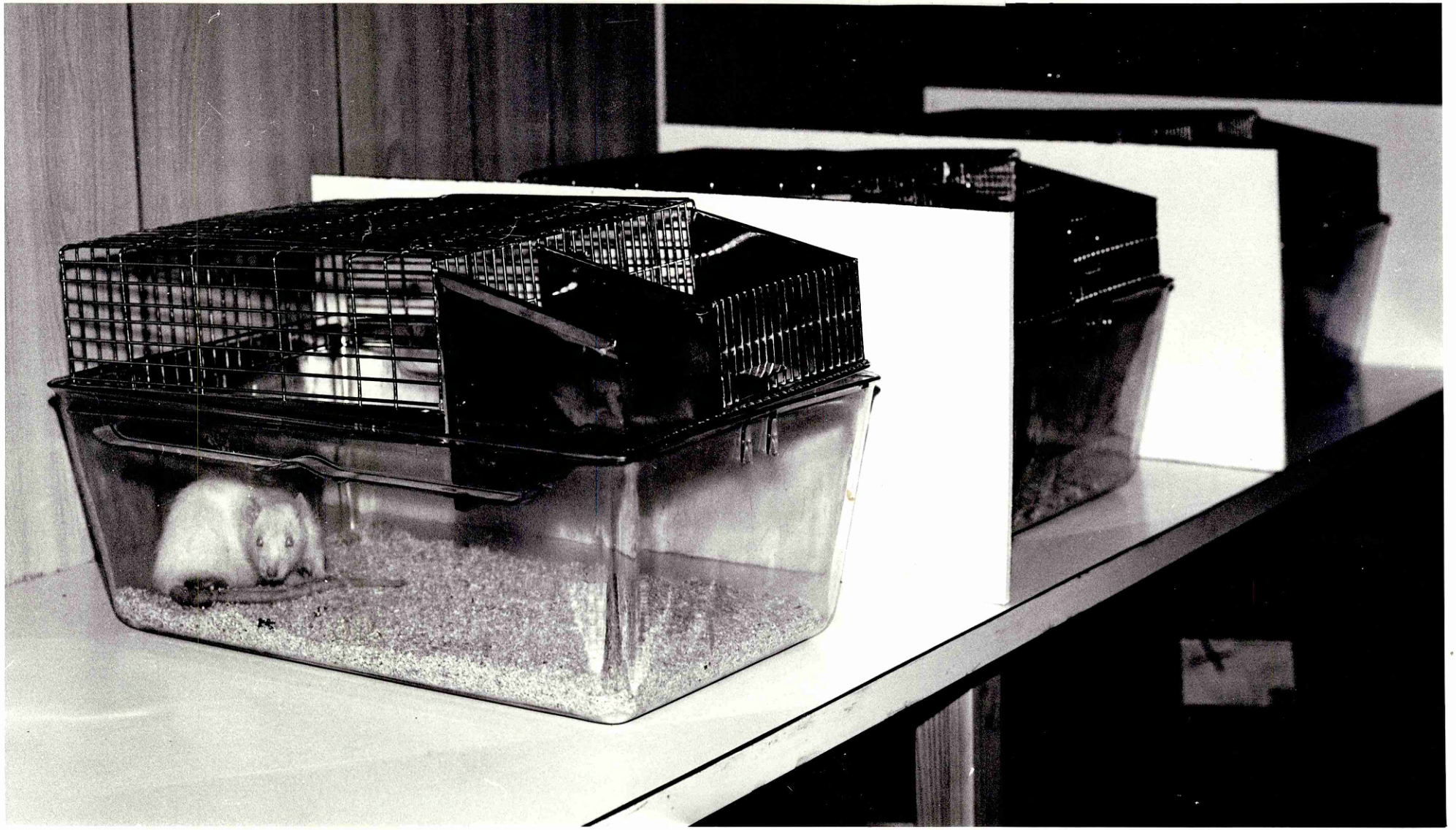


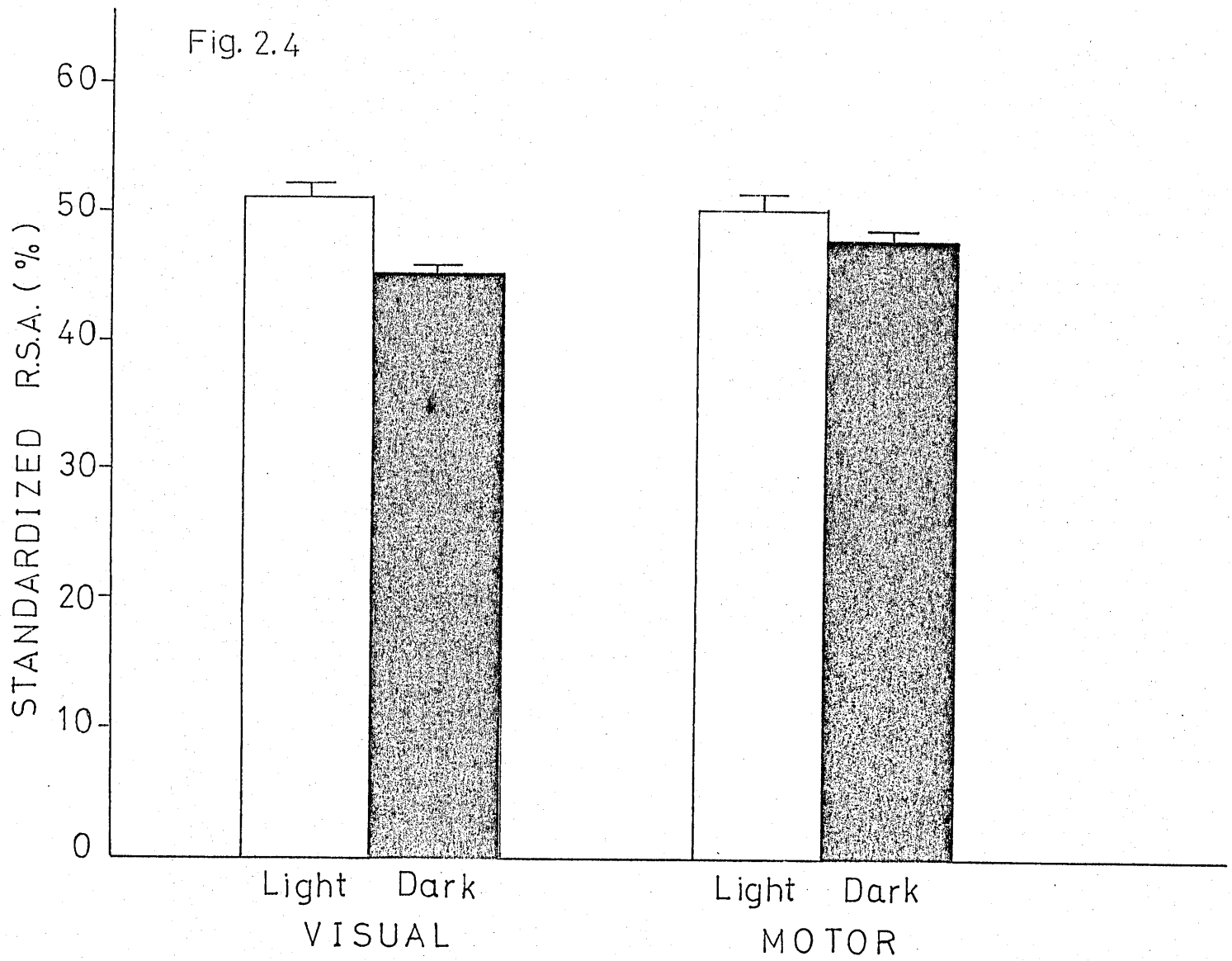
Plate 2. Experimental conditions during light exposure



Legend to Figures 2.4 and 2.5

Incorporation of ^{3}H lysine in visual and motor cortex of light exposed (white bars) and dark control (dark bars) littermate animals. 50 day old dark reared rats were injected intraperitoneally with 50 µCi ^{3}H lysine and killed after one hour of exposure to their respective conditions. Fig. 2.4 represents the means ± S.E.M. of the RSA's and Fig. 2.5 the means ± S.E.M. of the SA's, in each case standardized to a mean common to 3 experiments comprising a total of 13 animals (6 light exposed, 7 dark control). Visual cortex RSA L/D = 1.10 ($t = 4.468$, $p < 0.001$).

Fig. 2.4


STANDARDIZED R.S.A. (%)
60
50
40
30
20
10
0
Light
Dark
VISUAL
Light
Dark
MOTOR

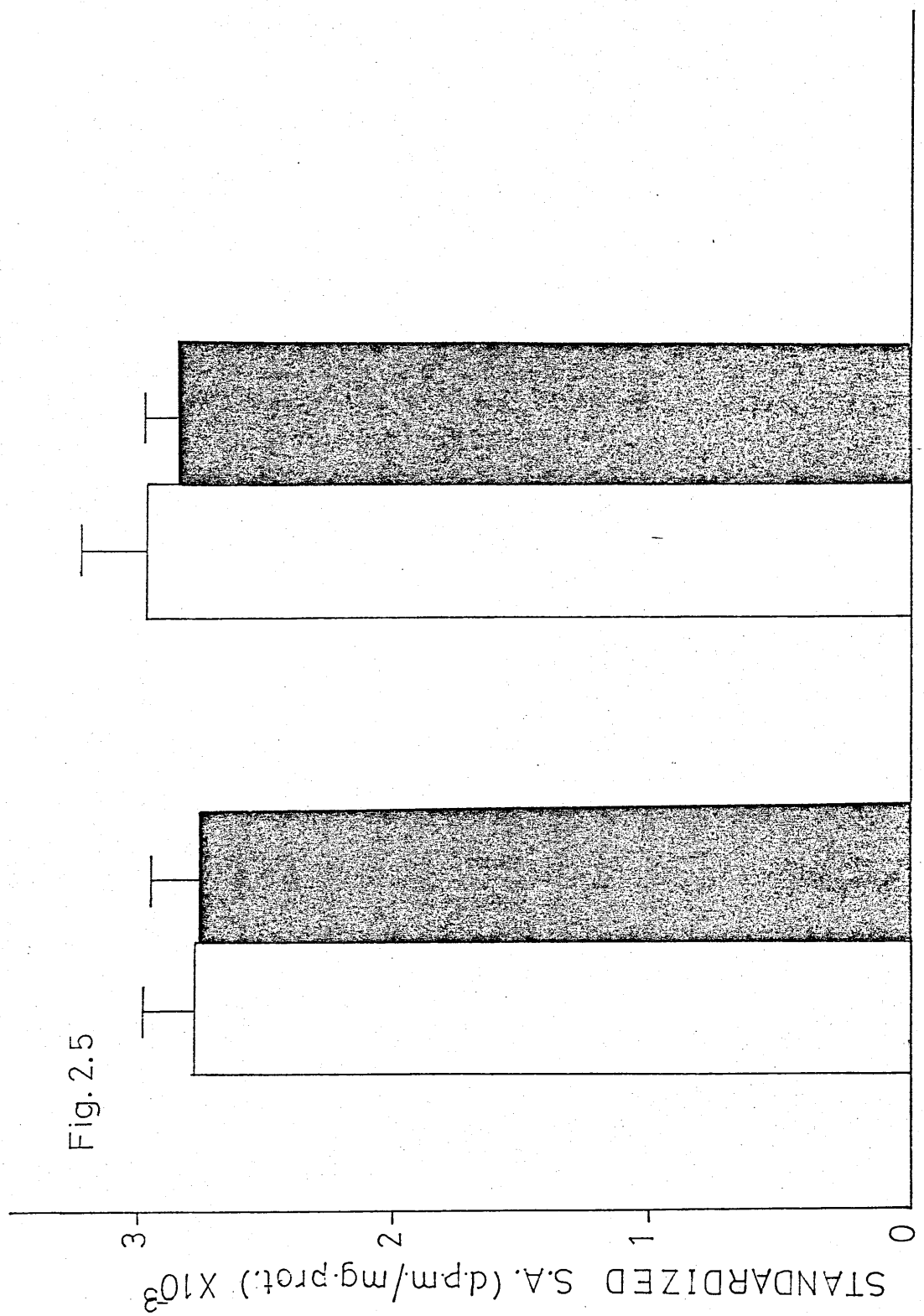

STANDARDIZED S.A. (d.p.m./mg.prot.) X10³
0
1
2
3
Light Dark
VISUAL
Light Dark
MOTOR

Fig. 2.5

= Y, then normalised SA = XY/150), in order to correct for variations in systemic distribution and uptake. Three separate experiments were performed, using a total of 13 animals. As there was some variation between the litters, the mean values of RSA and SA for each experiment/litter were calculated and corrected to equate with the mean of all the experiments combined, by scaling the values of individual animals accordingly. This procedure (e.g. Rose, 1967), while reducing the interlitter variation, does not systematically alter the size of differences between conditions (L and D) represented within each litter. There is a small (10%) but significant ($t = 4.468$, D.F. = 11, $p < 0.001$) elevation of incorporation calculated on the standardized RSA measure in the visual cortex only of the light exposed animals (see Fig. 2.4).

The exposure conditions represented in these experiments are comparable to those of Richardson and Rose (1972) who reported an elevation of 8.4% (sig. $p < 0.005$) in visual cortex RSA of animals at this age exposed to light for one hour, and which had increased to 17% (sig. $p < 0.01$) after a two hour period of exposure. These RSA results can therefore be regarded as a close replication under similar conditions. This increase is not reflected in the specific activity data reported here (Fig. 2.5). As can be noted, even with the standardized figures there exists some considerable variability between individual animals reflected in the larger standard errors here. In these experiments this may have been sufficient to mask the small but expected similar increase in SA incorporation. It should be noted in this context that Richardson and Rose (1972) reported a 12.1% (sig.$p < 0.01$) increase in visual cortex SA measures after a one hour period of exposure but which had fallen to a

(non-significant) increase of 6.3% after two hours. Alternatively, the discrepancy between the RSA and SA results reported in this present experiment may indicate that this increase is related to changes in precursor pool sizes.

It is an assumption and motivation of this thesis that the extent and nature of the stimulus-triggered neurochemical responsesto visual experience are state-dependent and vary with a complex of factors, some of which are discussed fully in later chapters. Behaviourally, these can be summarized as circadian factors (Richardson and Rose, 1971; Wood and Rose, 1979a)and levels of behavioural - and presumably cortical - arousal. This latter may be related to non-specific effects,perhaps involving differences in light intensity, ambient temperature and noise levels during exposure to the new visual experience.

To test this assumption, a similar set of experiments to the ones reported above was performed using identical procedures save that the exposure occurred under different conditions, including a lower overall noise level and in dim artificial illumination (approximately 50 lumens/m^2). Normal animals were also used in these experiments. Animals were exposed in a room of low wall reflectivity (dark blue) to a single overhead tungsten light source located at a distance of approximately 2 m from the experimental bench. Five separate experiments were undertaken here, using a total of 32 animals (13 light exposed, 11 dark controls, 8 normals).

Figs. 2.6 and 2.7 represent graphically the means ± S.E.M. of the results of these experiments. In visual cortex, the dark reared animals show 19-23% less incorporation compared to the normally reared animals, and in the motor cortex the incorporation is some 14-16% less (sig. in visual cortex RSA,

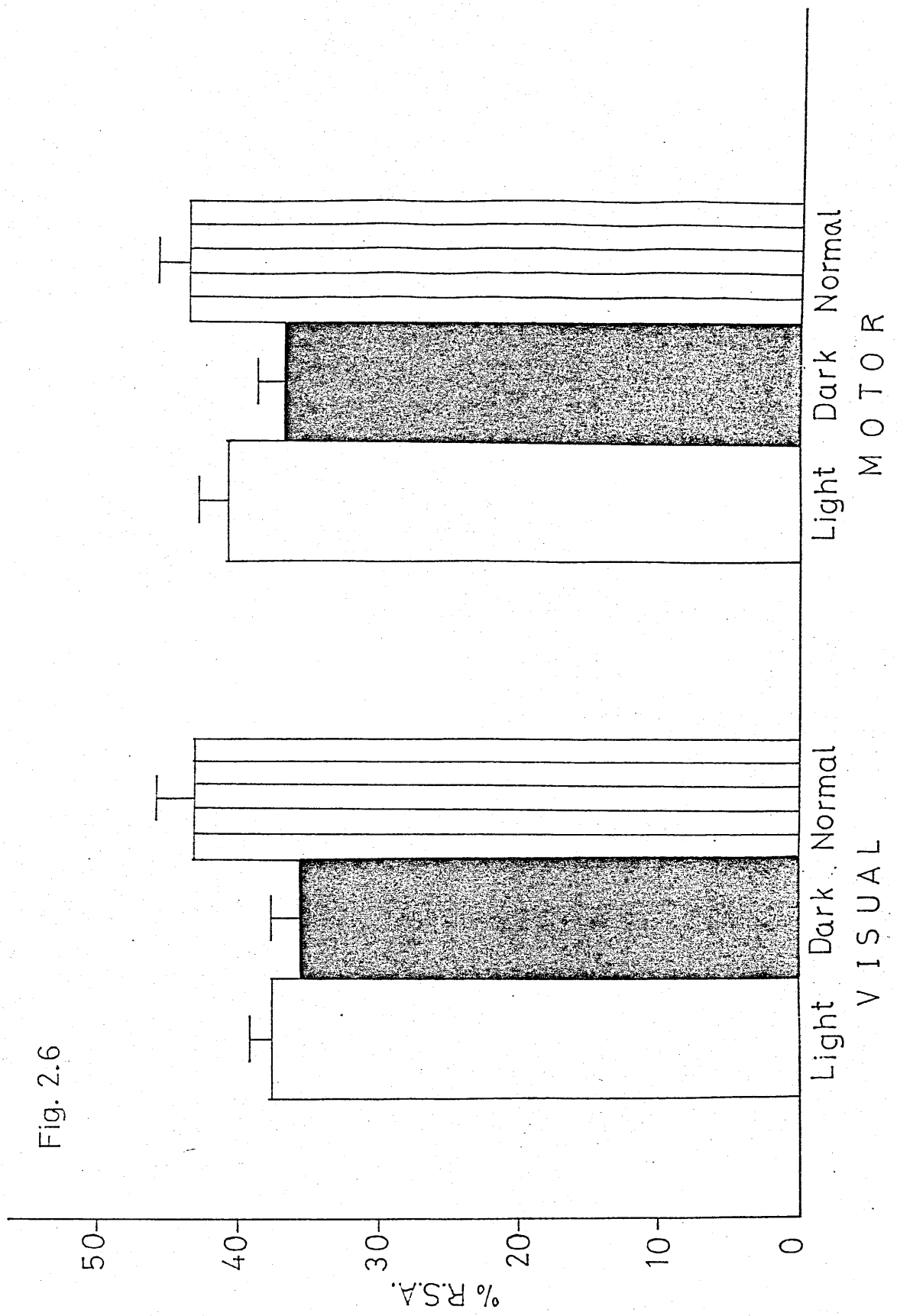
$t = 2.13$, D.F. = 17, $p < 0.05$; SA, $t = 2.45$, D.F. = 17, $p < 0.05$). These results can be interpreted as a lowering of the rate of protein synthesis, particularly in the visual cortex as a result of functional inactivity in the visual system over an extended period.

It will be noted, however, that although there is a small tendency to an increase in incorporation in both the SA and RSA measures of the light exposed animals, this does not approach statistical significance. This difference with the results of the previous experiment (Fig. 2.4) was attributed to the altered conditions of exposure. In this context it can be noted that previous findings from this laboratory (Rose, Sinha and Broomhead, 1973) indicated that the biphasic nature of the initial transient elevation of amino acid incorporation followed by a prolonged period of depression of incorporation is radically altered (the depression phase is absent) by exposure to more subdued lighting in an isolation room. The shift from a peak incorporation rate 3 h after the commencement of exposure (Rose, 1967) to one observed after 1 h (Richardson and Rose, 1972) is perhaps also an indication of the dependence of the highly sensitive and interactive biochemical processes responsive to changes in the environment or in the behavioural state of the animal on particular quantifiable differences in exposure conditions.

An important question which remained unanswered was to what extent do the effects of dark rearing and subsequent light exposure interact with ongoing developmental processes? One interpretation of visual deprivation/stimulation experiments is that they artificially delay the appearance of a normally

Legend to Figures 2.6 and 2.7

Incorporation of ^{3}H lysine in light exposed (light bars), dark control (dark bars) and normal (striped bars) littermate animals. 50 day old rats were injected intraperitoneally with 50 µCi ^{3}H lysine and killed after one hour of exposure to their respective conditions. Fig. 2.6 represents RSA's (visual cortex $N/D^{-} = 1.19$, $t = 2.13$, $p < 0.05$) and Fig. 2.7 represents SA's (visual cortex $N/D = 1.23$, $t = 2.45$, $p < 0.05$). The results in each case depict the means ± S.E.M. of 5 experiments (13 light exposed animals, 11 dark control and 8 normals).


% R.S.A.
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10
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Light
Dark
Normal
VISUAL
Light
Dark
Normal
MOTOR

Fig. 2.6

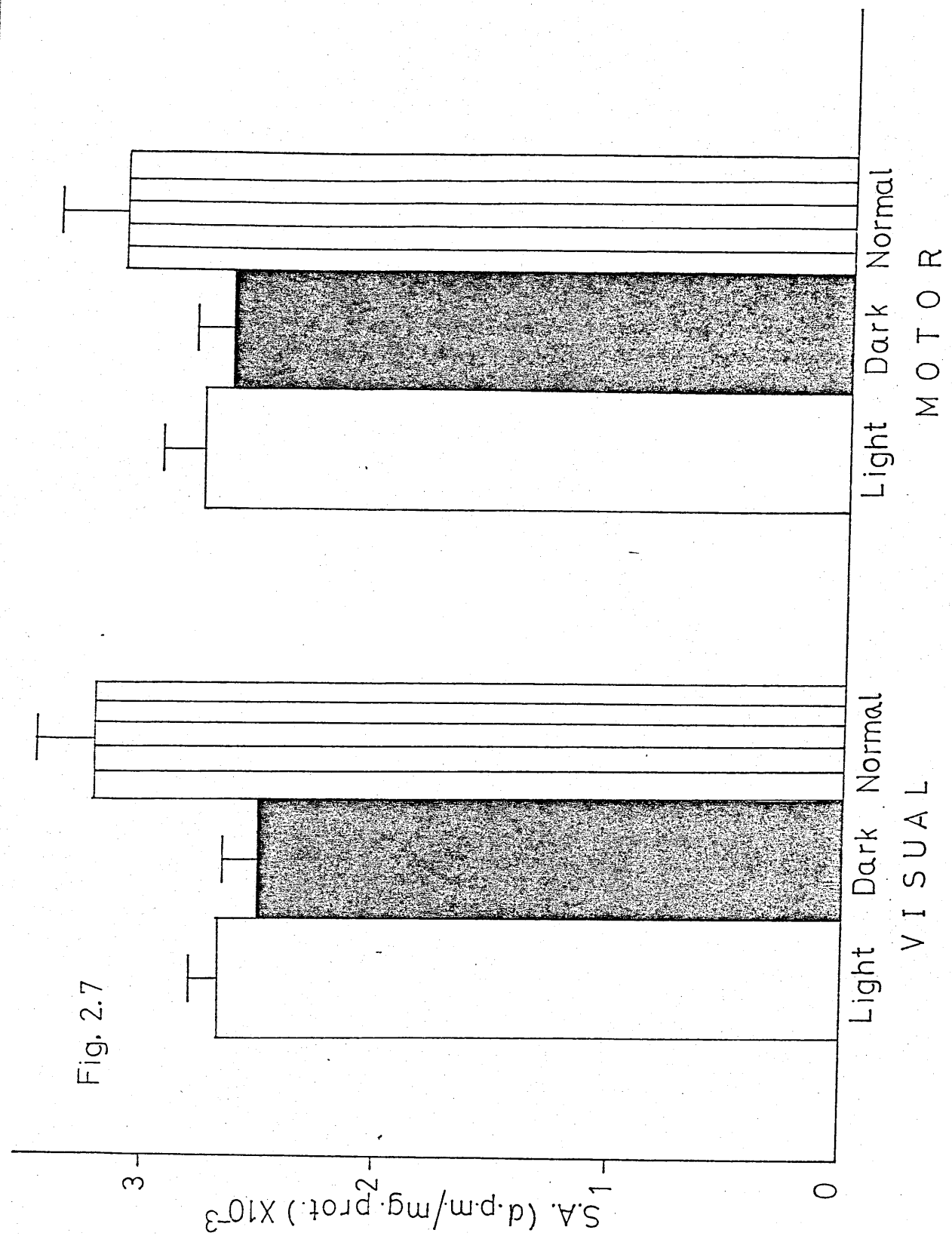

S.A. (d·p.m./mg·prot.) X10⁻³
0
1
2
3
Light
Dark
Normal
VISUAL
Light
Dark
Normal
MOTOR

Fig. 2.7

occurring developmental process, which would naturally appear on initial visual experience in the albino rat at around 14-16 days postnatally, with parting of the eyelids. This explanation probably assumes a greater plasticity of developmental mechanisms in the rat than has so far been demonstrated (Bigl and Biesold, 1978), the 50 day old animal having largely completed cerebral (Benjamins and McKhann, 1972) and behavioural (Barnett, 1975) processes of maturation.

Nevertheless, the well documented responses to light exposure in the 50 day old animal had not been demonstrated to occur at different ages. This would seem important in respect of attempts to relate the neurochemical changes directly to the substrate of the learning phenomena which may (indeed, must) be occurring during initial visual experience, and which would not be assumed to be unique to the seven week old animal. In relation to this, it had been demonstrated (Dewar, Reading and Winterburn, 1973; Dewar and Winterburn, 1973) that the incorporation of ^{14}C orotic acid into RNA in the visual cortex is enhanced during initial visual experience in a strain of 21 day old rats. These experiments can be regarded as pertinent to the present ones, in that apart from the similarity of the behavioural treatment, increased synthesis of RNA as the template for protein synthesis would be expected. In addition, it seemed sensible to exploit the known higher rates of cerebral protein synthesis in younger animals (Miller, 1969).

Two age points were chosen for these experiments, around the age of conventional weaning (21 days) and normal eye opening (c. 15 days). It could not be assumed that the optimal time period of radioactive pulse and/or light exposure would be in any way comparable to that in the mature animals, and the problem was approached initially by running a time course of incorporation in normal 20 day old animals.

Twelve animals were used in this initial experiment. The procedure was essentially similar to that described earlier for the 50 day old animal with the following differences. Animals used were 20 days of age and weighed 40 ± 7 g, to which figure specific activity results were corrected as described earlier. Animals were injected with 25 µCi of ^{3}H lysine.

As before, three animals were used at each time period of 15, 30, 60 and 120 minutes. Figs. 2.8 and 2.9 illustrate the means ± S.E.M. of these results. Incorporation increases very rapidly over the initial hour to reach 53% RSA and an SA figure of 8000. These can be compared with the figures of 33% and 3000 obtained with the 50 day old animals after one hour with twice the quantity of ^{3}H lysine injected. Like the results obtained with the older animals however, only a minimal further increase was observed between the first and second hour post-injection. Indeed, with the specific activity measure at least, the rate of increase has rapidly declined after the initial 30 minutes.

Two time periods were chosen for exposure of the weanlings to light, both using a simultaneous pulse of 25 µCi ^{3}H lysine, with other procedures exactly as described for the experiments illustrated in Figs. 2.4 and 2.5 (open laboratory exposure conditions). As dark reared female littermates were available at this age, it was decided to use these in addition to the male animals. Routinely, these had been excluded from our normal experimental design after Richardson (1974) had observed that although the mean level of incorporation (RSA) of ^{3}H lysine after a one hour exposure period was very similar to that of the male animals, the variance in the results was some 6-fold greater with the 50 day old female animal. This may be

Legend to Figures 2.8 and 2.9

Time course of incorporation of ^{3}H lysine in visual (broken line) and motor (solid line) cortex of 20 day old normally reared rats. Animals were injected intraperitoneally with 25 µCi ^{3}H lysine and incorporation determined at the times shown. Fig. 2.8 depicts the RSA's and Fig. 2.9 the SA's. Each point represents the means ± S.E.M. of 3 determinations.

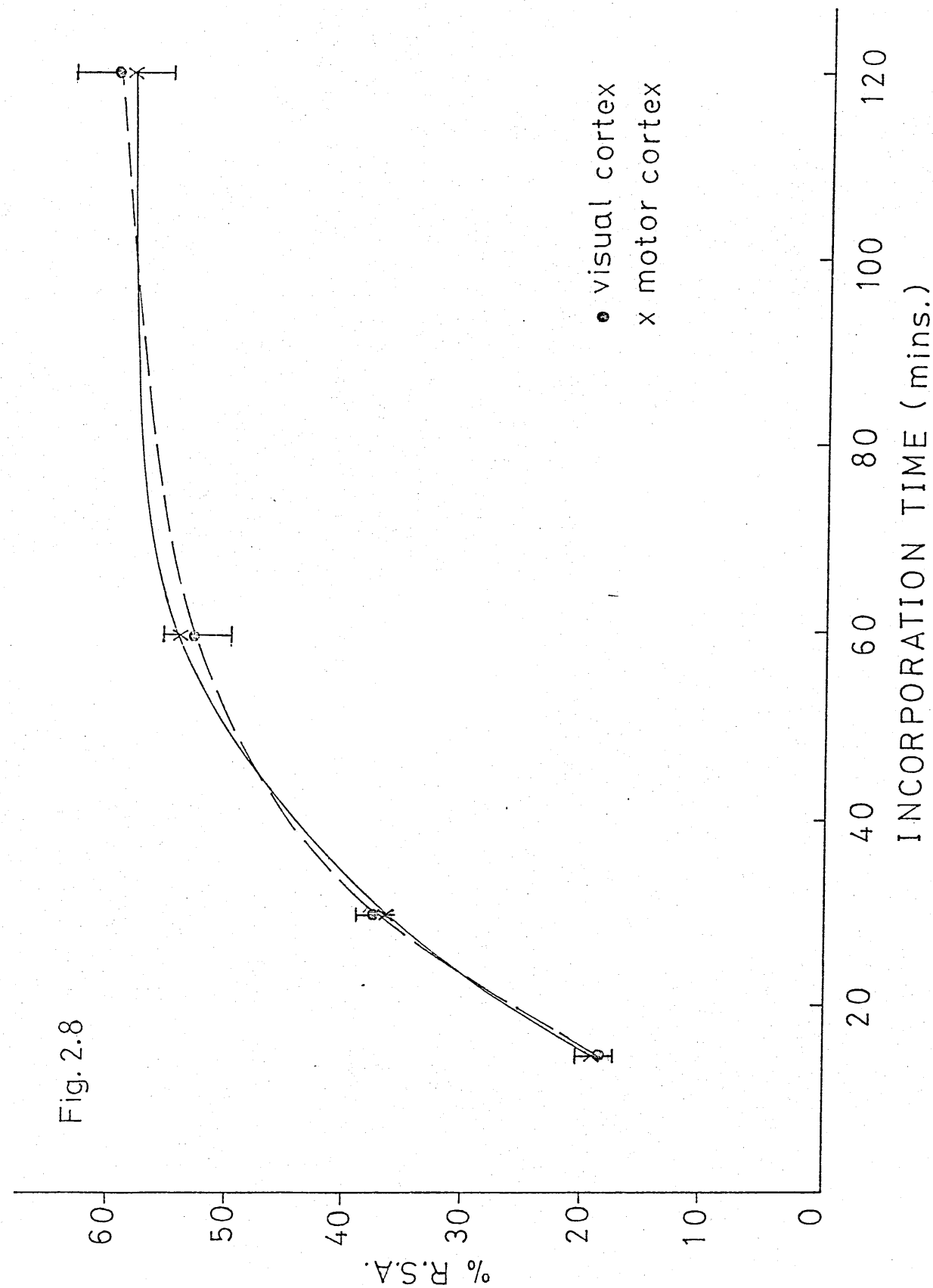

% R.S.A.
60
50
40
30
20
10
0
20
40
60
80
100
120
INCORPORATION TIME (mins.)
• visual cortex
x motor cortex

Fig. 2.8

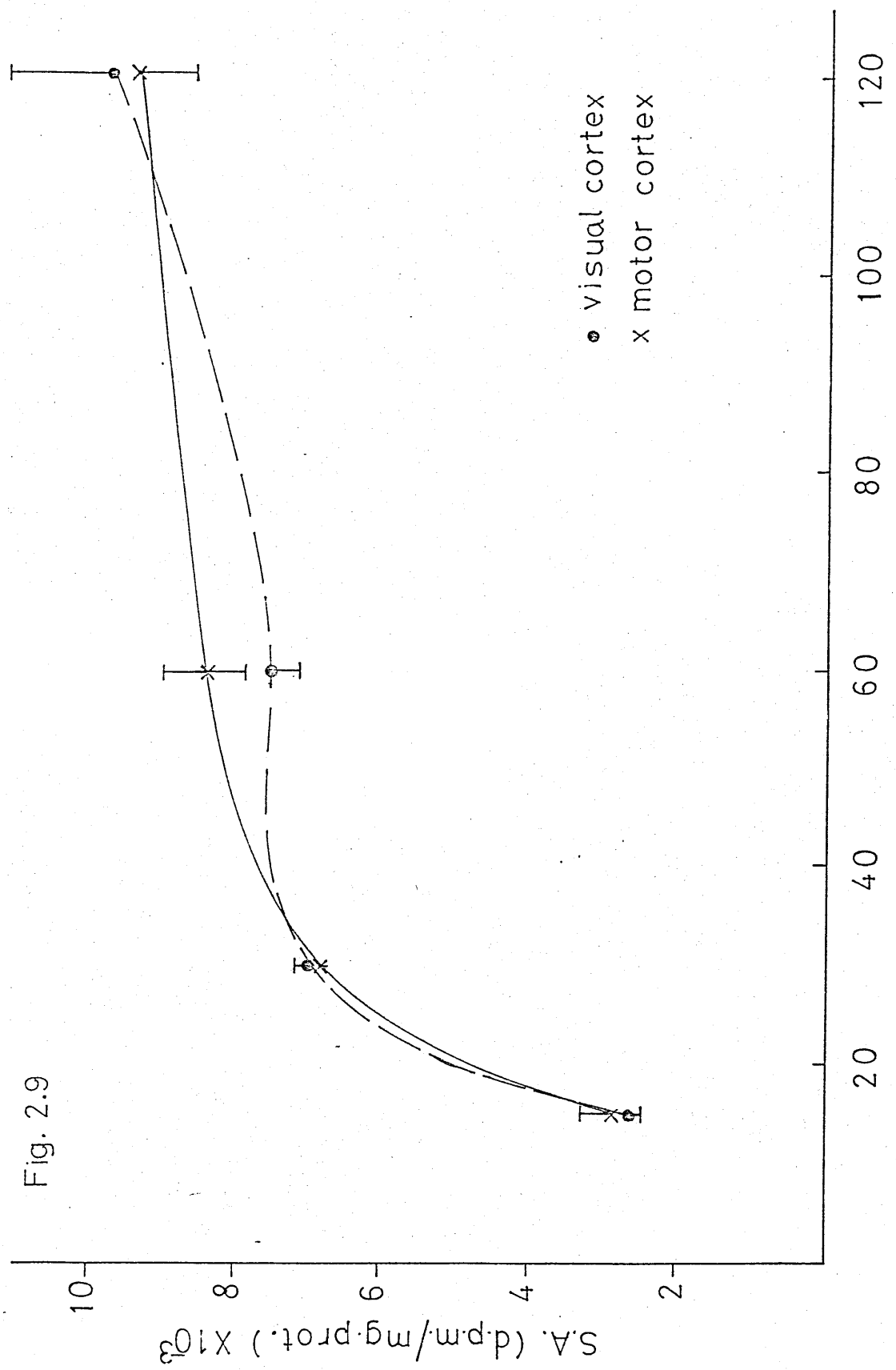

S.A. (d.p.m./mg.prot.) X10³
10
8
6
4
2
20
40
60
80
100
120
INCORPORATION TIME (mins.)
• visual cortex
x motor cortex

Fig. 2.9

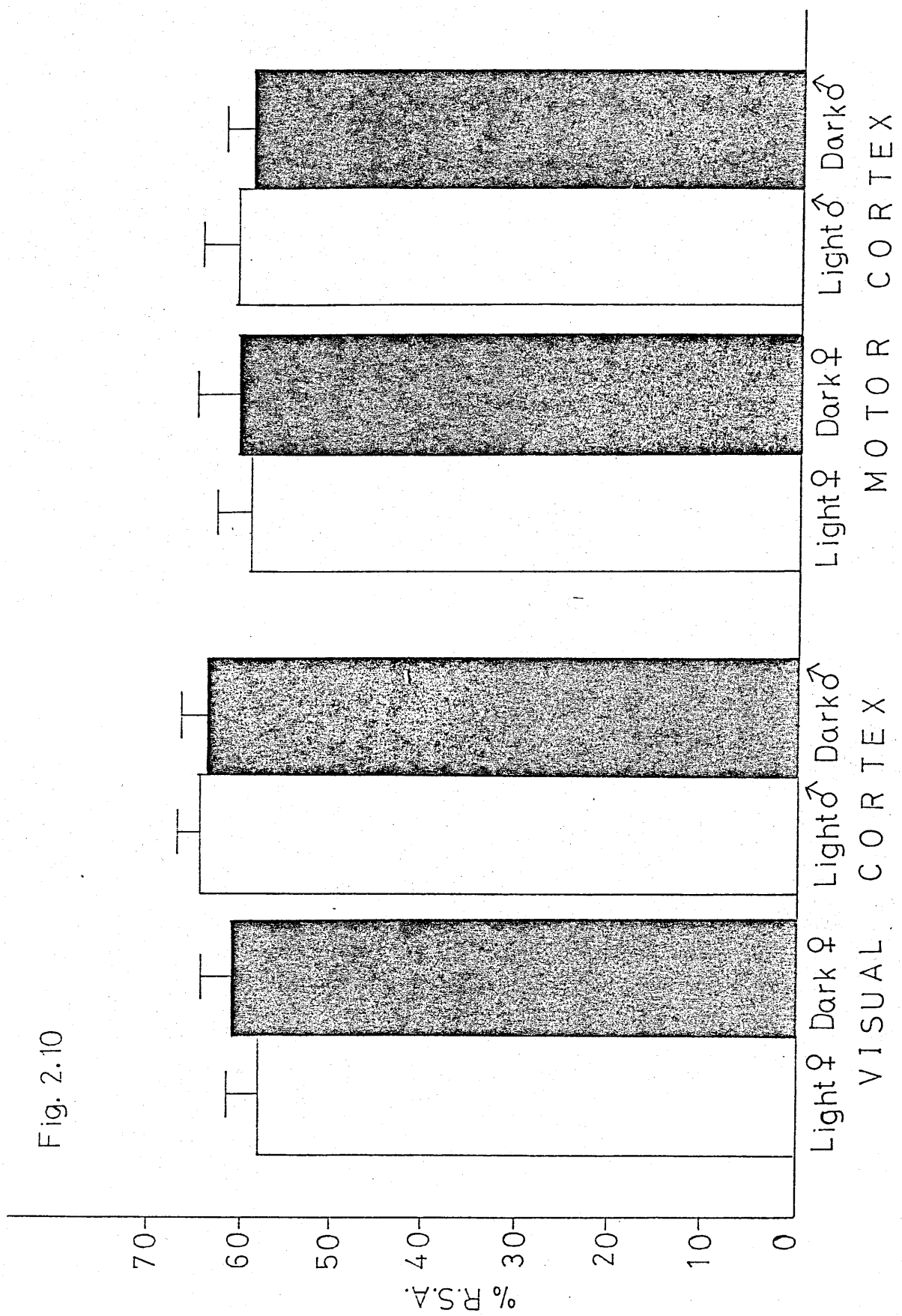
related to differences in circulating hormones, some of which become particularly active at this age as sexual maturity is approached, and which may affect the rate of uptake. It was assumed that these differences would not be as prevalent at weaning, and accordingly all littermate animals were used. The time periods used for these experiments were 60 mins (results ± S.E.M. illustrated in Figs. 2.10 and 2.11) and 30 mins (Figs. 2.12 and 2.13). Separate results are presented for the male and female animals in each group. Figs. 2.10 and 2.11 represent the results obtained with a total of 20 animals (6 light exposed and 6 dark control males, and 4 light exposed and 4 dark control females). Figs. 2.12 and 2.13 represent the results with a total of 13 animals (3 light exposed males and 4 light exposed females and 3 dark control animals of each sex).

Although these experiments must be regarded as initial exploratory attempts, using small numbers of animals in order to establish an optimal exposure/incorporation period, they provide no indication of any consistent pattern or trend to differences between the light exposed and dark control animals at this age. It would seem unwarranted to accept these preliminary results at face value however, in view of the reported elevation of ^{14}C orotic acid into RNA in the visual cortex of 21 day old light exposed animals (Dewar, Reading and Winterburn, 1973) and of soluble glycoproteins into visual and motor cortex of 21 day old animals (R.D. Burgoyne and S.P.R. Rose, unpublished).

One explanation which may account for this negative finding is that all animals used in these experiments were exposed between 14.00 and 16.00 hours. It has subsequently been shown

Legend to Figures 2.10 to 2.13

Incorporation of ^{3}H lysine in visual and motor cortex of light exposed (white bars) and dark control (dark bars) 21 day old male and female littermate rats. Animals were injected intraperitoneally with 25 µCi ^{3}H lysine and killed after 60 minutes (Figs. 2.10 and 2.11) and 30 minutes (Figs. 2.12 and 2.13) of exposure to their respective conditions. The results represent the means ± S.E.M. of from 3 to 6 animals in each condition.


% R.S.A.
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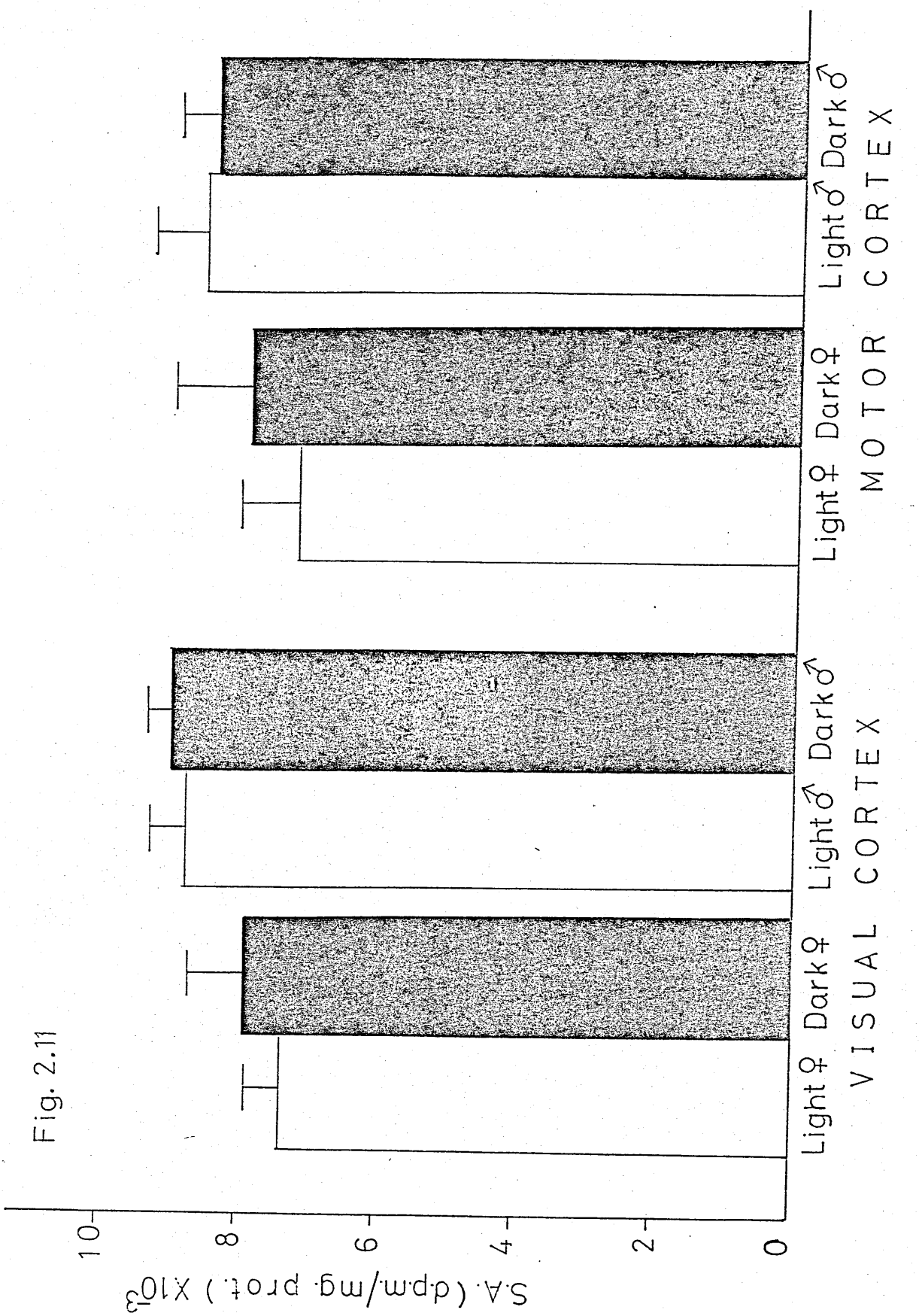
60
70
Light♀
Dark♀
Light♂
Dark♂
VISUAL CORTEX
Light♀
Dark♀
Light♂
Dark♂
MOTOR CORTEX

Fig. 2.10


S.A. (d.p.m./mg. prot.) X10³
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4
6
8
10
Light♀
Dark♀
Light♂
Dark♂
VISUAL CORTEX
Light♀
Dark♀
Light♂
Dark♂
MOTOR CORTEX

Fig. 2.11

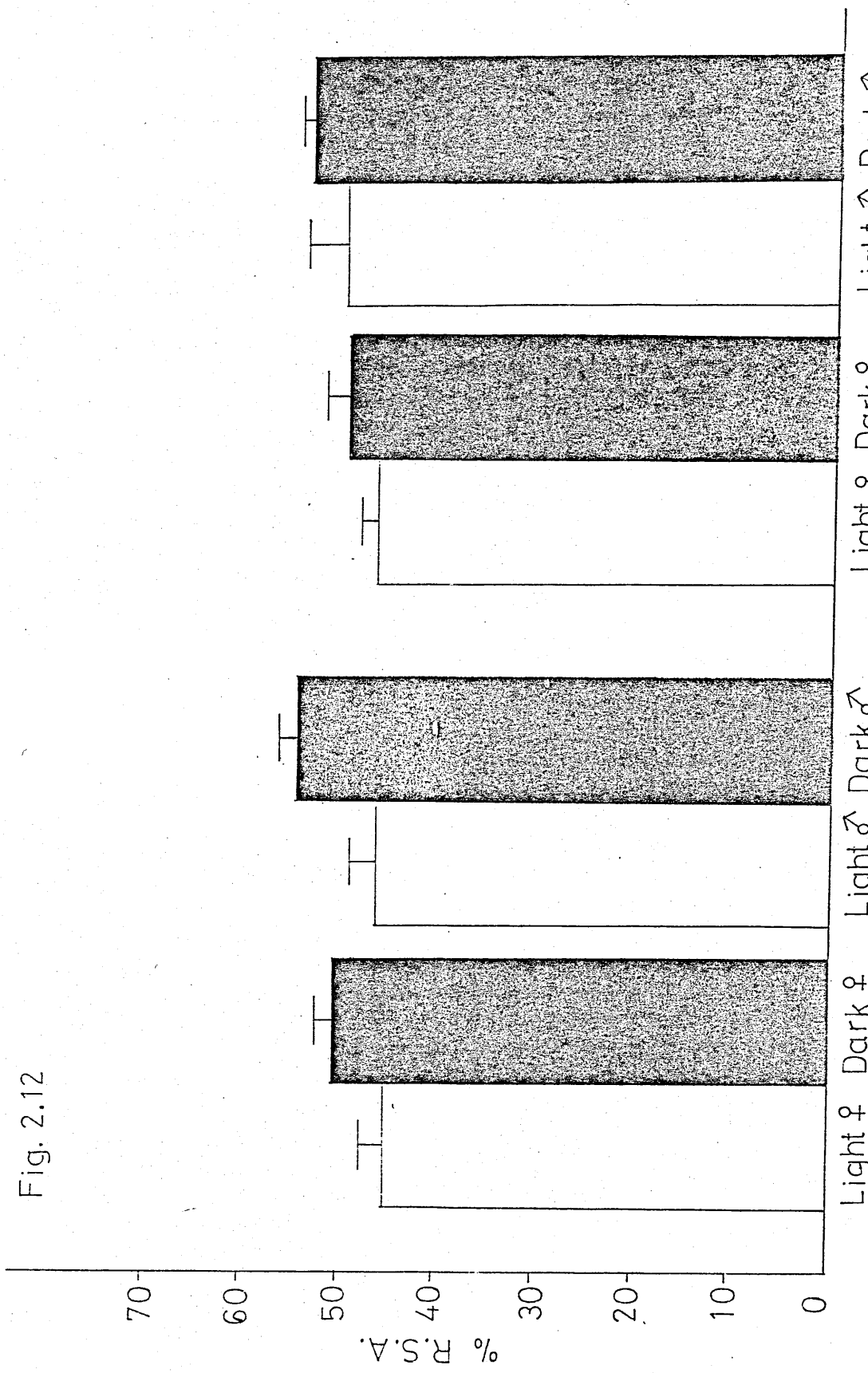

% R.S.A.
0
10
20
30
40
50
60
70
Light ♀ Dark ♀ Light ♂ Dark ♂
VISUAL CORTEX
Light ♀ Dark ♀ Light ♂ Dark ♂
MOTOR CORTEX

Fig. 2.12

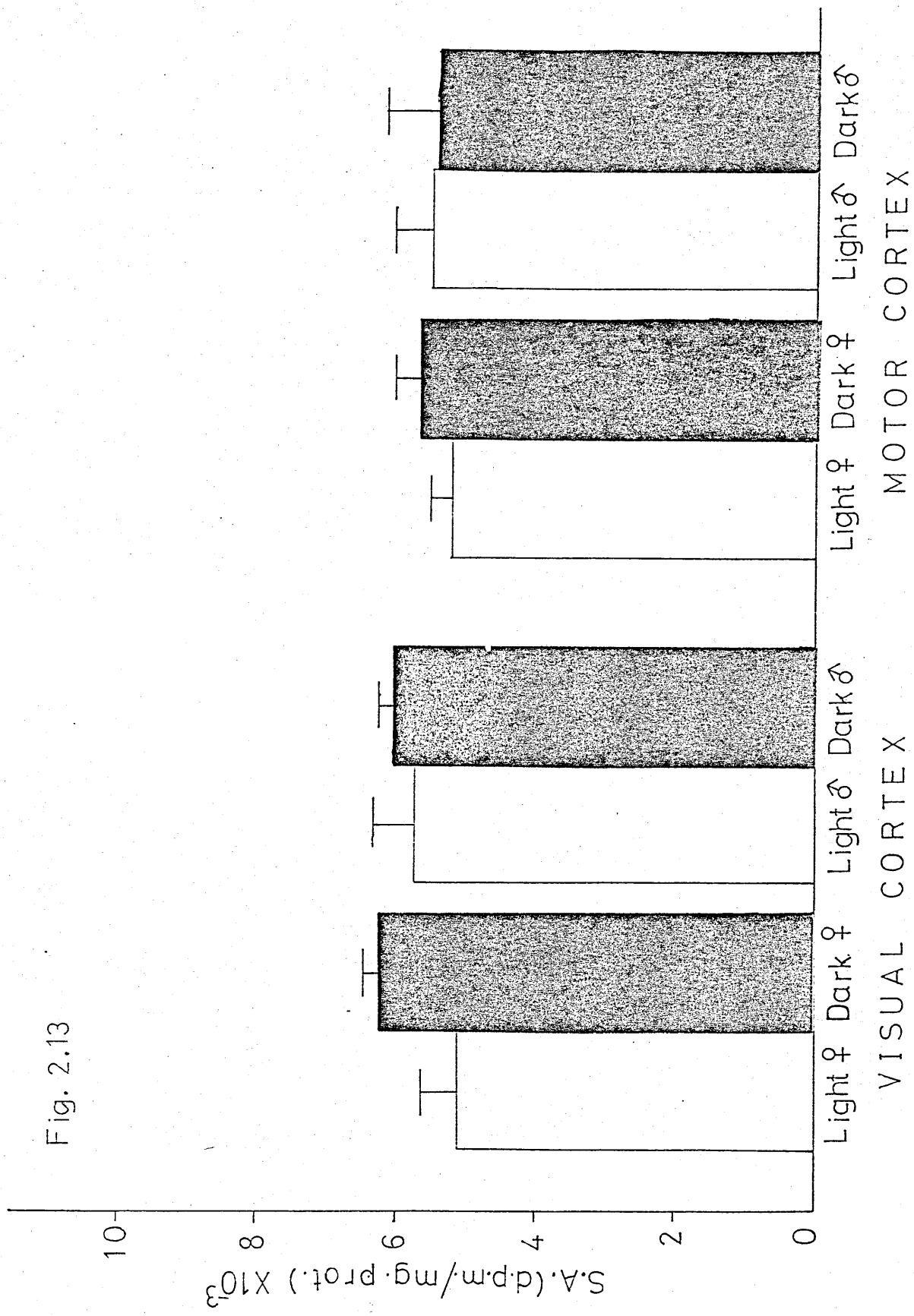

S.A.(d.p.m./mg.prot.) X10⁻³
0
2
4
6
8
10
Light ♀
Dark ♀
Light ♂
Dark ♂
VISUAL CORTEX
Light ♀
Dark ♀
Light ♂
Dark ♂
MOTOR CORTEX

Fig. 2.13

(Wood and Rose, 1979a) that,at least as far as AChE is concerned, the stimulus induced elevations are much reduced or absent if exposure occurs late in the day, and that this is probably related to the circadian rhythm of behaviour which persists even in the dark reared animal. The implicationsof these findings are discussed further in Chapter 5 of this thesis. These results do indicate, however, that the variability of the data obtained with female animals (as deduced from the S.E.M's)is only marginally greater than the values for males at this age, and justifies their inclusion.

It was also decided to use some animals around the age of natural eye opening. A litter was selected in which ten animals (5 light exposed, 5 dark control) from a total litter of 14 had just completed eye opening (14-15 days post birth). Animals were injected with 25 µCi ^{3}H lysine and initially exposed for 30 minutes to laboratory illumination before administration of a pulse of radioactivity simultaneously with an additional 30 minutes of exposure. Specific activities were corrected to 25 g. All other procedures were as described previously. The means of these results ± S.E.M. are illustrated in Figs. 2.14 and 2.15. A large increase of incorporation in both brain regions of the light exposed animals is evident in the RSA results (54% in visual cortex, sig. $t = 3.70$, D.F. = 8, $p < 0.01$ and 49% in motor cortex, sig. $t = 2.95$, D.F. = 8, $p < 0.02$) and in the SA results (53% in visual cortex, sig. $t = 2.99$, D.F. = 8, $p < 0.02$ and 57% in motor cortex, sig. $t = 3.17$, D.F. = 8, $p < 0.02$). Although these results, obtained with a total of only 10 animals, cannot be extrapolated to a general figure, they can be regarded as

Legend to Figures 2.14 and 2.15

Incorporation of ^{3}H lysine into visual and motor cortex of light exposed (white bars) and dark control (dark bars) 15 day old littermate rats. Animals were injected intraperitoneally with 25 µCi ^{3}H lysine and killed after 30 minutes of exposure to their respective conditions. The light exposed animals were prior exposed to light for 30 minutes. Fig. 2.14 depicts the RSA's (L/D visual cortex = 1.54, $t = 3.7$, $p < 0.01$; motor cortex = 1.49, $t = 2.95$, $p < 0.02$) and Fig. 2.15 the SA's (L/D visual cortex = 1.53, $t = 2.99$, $p < 0.02$; motor cortex = 1.57, $t = 3.17$, $p < 0.02$), and represents the means ± S.E.M. of 5 animals in each condition.

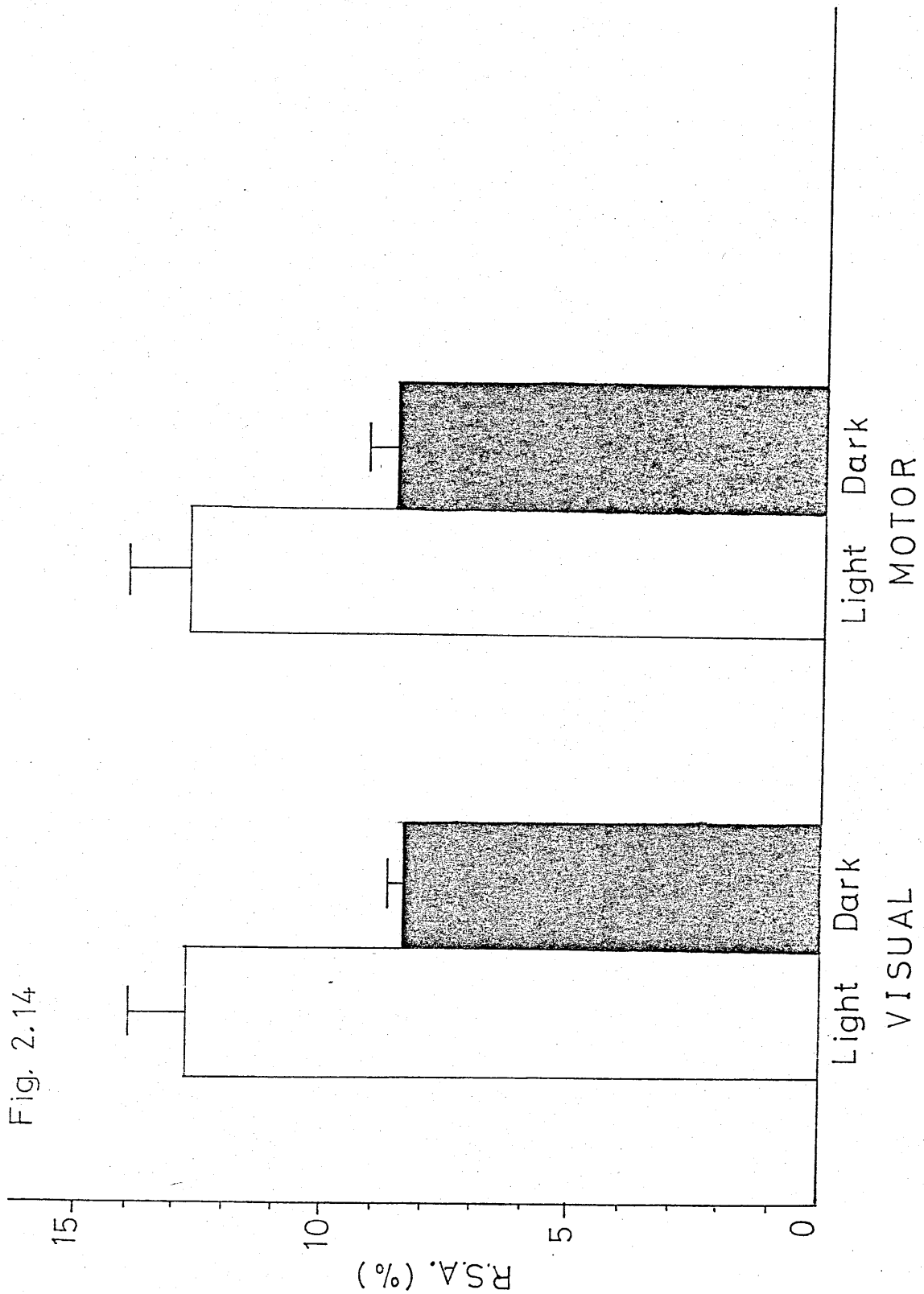
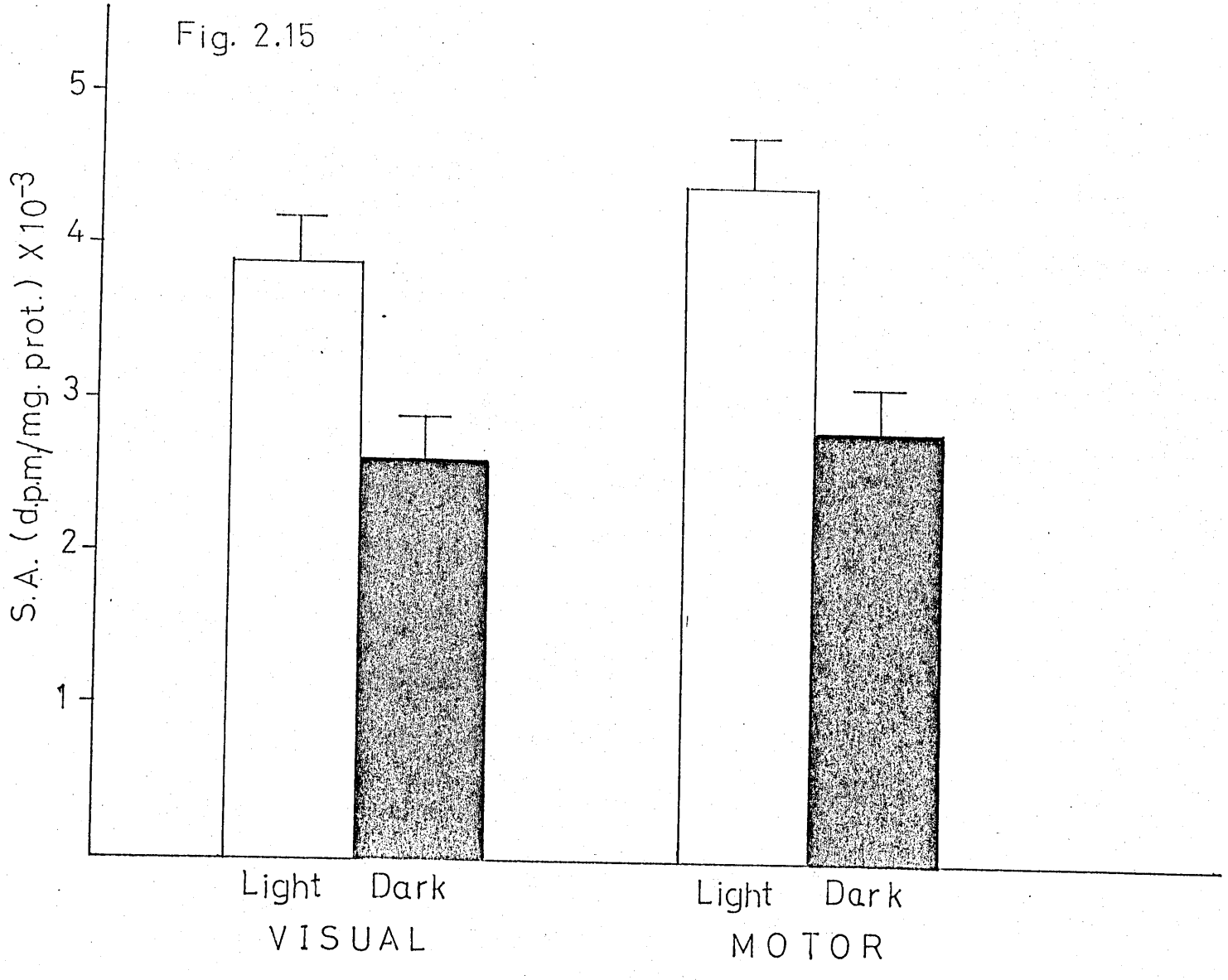


Fig. 2.14

Fig. 2.15



highly suggestive of a large increase in protein synthetic capacity at a significant developmental stage, but which is not regionally specific. It is interesting to note in this context that the elevation of incorporation into a soluble glycoprotein fraction recently observed in 21 day old animals occurs into the visual and motor cortex (R.D.Burgoyne and S.P.R. Rose, unpublished), whilst the increase in particulate glycoprotein in the 50 day old animal is regionally specific (Burgoyne and Rose, 1978).

It was decided , in order to pursue the main aims of this project, not to investigate further this phenomenon, which may be interpreted as a 'programmed' response interacting with an environmental stimulus. It had recently been shown (Sinha and Rose, 1976) that large changes of up to 30% in enzymes of the ACh system were detectable in animals exposed for 3 hour periods after 50 days dark rearing, and it was decided to pursue these biochemical markers to relate to the main aims of this project.

Conclusions

Time courses of incorporation into acid precipitable proteins of the visual and motor cortex of normal 50 day old animals confirmed previous results, and new data was presented on the time course of incorporation in 20 day old animals. The light induced elevation of incorporation after a one hour period of exposure of 50 day old dank reared animals to the light was replicated, but on the RSA measure only. No light induced elevation of incorporation was detectable in 21 day old animals, although these negative results were not regarded as conclusive. Preliminary results indicated a large increase of incorporation in both the visual and motor cortex of light exposed animals at the age of natural eye opening.

CHAPTER THREE

CHOLINERGIC SYSTEMS, EXPERIENCE AND LEARNING

From the considerations outlined in Chapter I, it has been assumed that the occurrence of biochemical changes other than simply synthesis or turnover of macromolecules might be involved in the adjustment to new experience, especially during the initial phases of exposure to the new visual experience, and that this may involve transmitter systems underlying a synaptosomal regulation of synaptic connectivity. One such candidate is undoubtedly acetylcholine. Although the role of ACh as a central mammalian neurotransmitter is not so firmly established as its role at peripheral synapses, the ACh content in general has been shown to vary inversely with the degree of functional activity of the mammalian brain (Hrdina, 1974), and is present in significant amounts. This would be expected if neuronal activity is associated with an increased release of ACh from the synaptic terminal followed by its hydrolysis by cholinesterase.

The concentration of ACh in the brain of the rat has been shown to exhibit a circadian rhythmicity (Hanin et al., 1970; Saito, 1971) in common with some other biogenic amines. Furthermore there is a reasonable correlation between the amounts of transmitter and the enzymes CAT and AChE found in most brain regions, with the exception of the cerebellum. More than 70% of ACh present in the forebrain is in a synaptosomal fraction (Hebb and Whittaker, 1958), about half of which can be recovered in a vesicular fraction (Whittaker, 1959). The synaptosomal fraction also contains some 50-70% of the total CAT activity found in brain homogenate (Hebb and Smallman, 1956).

Electrophysiological stimulation of the mesencephalic reticular formation (Kanai and Szerb, 1965; Phillis, 1968) the geniculate bodies (Collier and Mitchell, 1966; Neal et al., 1968) as well as direct stimulation of the cortex (Celestia and Jasper, 1966; Phillis, 1968) has been shown to result in large increases in released ACh from the cerebral cortex, including the primary receiving areas, in the anaesthetised cat. Visual stimulation by continuous light for 1 hour in atropinised and anaesthetised rabbits has been shown to result in increases in ACh from the visual cortex, and a smaller increase from other cortical areas (Collier and Mitchell, 1966), while Bartolini et al. (1972) found an increased release of ACh from the cerebral cortex of the anaesthetised cat following photic stimulation. The possibility exists that this was due to an activation of the diffuse cholinergic arousal system, although the existence of a specific thalamo-cortical cholinergic pathway to the primary sensory receiving areas, in addition to diffuse reticulo-cortical and septo-cortical pathways ascending to the cortex has been proposed. There are also some indications, at least in the cat (Hebb et al., 1963) of the existence of some local cholinergic neurones in the cortex, which can remain viable in chronically undercut cortex.

In the visual system of the rat, Glow and(Sam) Rose (1964) reported that after 'irreversible inhibition' of AChE by DFP treatment, the de novo synthesis of the enzyme in the retina is partly dependent upon subsequent stimulation by light and proposed ACh as a retinal transmitter, while significantly lowered AChE levels in the retina of dark reared rats have been reported (Liberman, 1962). There is no specific evidence of a direct retino-geniculo-cortical cholinergic link in the rat. By

estimation of the specific cholinergic marker enzyme CAT, no decreases have been found after eyeball enucleation, lateral geniculate or visual cortical destruction (Bigl and Schober, 1977) although extensive neuronal degeneration occurred, and this is in accord with other pharmacological and physiological data.

The transient increases which have been observed in the enzymes of ACh metabolism (Sinha and Rose, 1976) and the muscarinic receptor (Stewart and Rose, 1978) in the visual cortex of the 50 day old dark reared rat following three hour periods of new visual experience may be a consequence of the result of acquisition processes or consolidation of experience, or may be a non-specific effect arising possibly from an increased activation of the cortical arousal mechanisms. A number of studies have implicated the effects of differential experience or of more controlled training situations, in modulating cholinergic synaptic activity in the rat brain. The experiments from the group of Bennett and Rosenzweig on the effects of rearing in enriched and impoverished environments (Rosenzweig et al., 1972; Rosenzweig and Bennett, 1975) are well known examples of the former type, indicating relatively long term plastic adjustment to differential experience. Rats which were reared from weaning for 30 days, or simply given 2 h of experience every day, in an enriched environment, displayed morphological changes in cells and small increases in the weight of cortex, compared to animals reared in impoverished environments. Increases of around 5-10% in total AChE, total ChE and in the ChE/AChE ratio were detected in the enriched experience animals, but a decrease in the AChE/mg tissue was reported. These effects were greatest in the occipital cortex. The ChE/AChE ratio change

was interpreted as due to changes in the rate of glial proliferation. These results do not appear to be specifically related to alterations in visual input or function however, as dark reared rats showed similar differences in the enriched and impoverished conditions (Rosenzweig et al., 1969). Unlike the AChE changes, CAT and ACh content increased proportionately to the increase in tissue weight (Bennett et al., 1968). A number of control experiments tended to eliminate these differences as being due to an accelerated maturation, differences in locomotor behaviour, handling effects, hormonal or stress effects, or mere visual exposure to the enriched environment without participation. However, some of these factors have been shown to influence cholinesterase levels in rat brain. McKinney (1970) investigated brain ChE in grouped and singly caged adrenal demedullated rats, and this treatment produced a decrease in the brain AChE content of the singly caged animals. Quay et al. (1971) have reported differences in the circadian rhythm of AChE in whole cerebral cortex related simply to caging singly or in groups.

Another report has indicated a much more dramatic effect arising from altered long-term visually mediated experiences on cortical specific AChE activity. Singh et al. (1967) found a 100% increase in AChE/mg protein in the posterior cortex of rats reared for 70 days with a pattern of stripes compared to control animals reared with a blank visual field, although this report has not been replicated.

A number of studies have investigated the action of cholinergically acting pharmacological agents directly on the acquisition and consolidation of trained behaviour. A number of early studies reported some facilitation associated with

pre- or post-trial injections of convulsant and anticholinesterase agents. Platt (1951) found an increase in acquisition rates of rats treated with the AChE inhibitor DFP, while Stratton and Petrinovich (1963) reported a facilitation of the rate of maze learning in rats following post-trial injection of eserine, and they suggested that the ratio of ACh/AChE was an index of the probable time course of perseverative neural activity. McGaugh and Thompson (1962) and Petrinovich (1963) reported the facilitation of successive or simultaneous discrimination learning with administration of strychnine sulphate. These type of results were regarded as supporting the idea of the perseveration of neural traces in a closed reverberatory system as a basis of short term storage (John, 1967) which then may become consolidated to form permanent memory.

A subsequent number of experiments by Deutsch (1966, 1971) indicated a complex series of events associated with administration of anticholinesterase and anticholinergic drugs, depending upon the time of injection before or after training. Rats were trained to avoid electric shock in a Y maze and injected intracerebrally with DFP. This treatment was reported not to affect the acquisition of this habit but to affect the savings on retesting (interpreted as a measure of strength of retention). Initial training to a criterion of 10 out of 10 correct responses followed by DFP injection after 30 minutes significantly lowered this retention, but not if injected 3 days later, and after this time interval retention again significantly became worse following DFP injection compared to controls. Subsequent experiments indicated that facilitation of an almost forgotten 28 day old habit could occur following DFP injection. Post synaptic receptor block with the anticholinergic scopolamine

produced results the opposite to that of DFP. Injection of the cholinomimetic carbachol blocked habits learned 7 days but not 3 days previously. As a consequence of these and similar findings, it was hypothesised that as a result of learning the post-synaptic endings at a specific set of synapses become more sensitive to transmitter with a specific time course of increase and decline.

Although some of these results have been repeated with appetitive rather than aversive tasks (Biederman, 1970) and with other routes of administration and inhibitors (Hamburg, 1967), it is not clear how these agents are acting selectively on the proposed memory synapses however. There seems little doubt that some of the contradictory results which have been obtained following injection of these agents may be related to sites of administration and dosage administered, and the assumption has been that DFP may facilitate cholinergic transmission if release of transmitter occurs, although increased pre-synaptic activity and release of larger amounts of ACh may lead to an accumulation such that a postsynaptic inhibition results. Atropine is known to alter the ratio of free/bound ACh (Crossland and Slater, 1967) and the physiological significance of this is obscure.

Another series of experiments have attempted to define the particular cholinergic systems involved in acquisition and storage, in addition to the role of the different compartments of the transmitter as a function of time following training. This was the approach of Matthies et al. (1974, 1976). Using a shock motivated brightness discrimination task, which took about 45 mins to attain criterion, the ACh content in several brain areas was determined at various times following the training procedure. A six-fold increase in free ACh content in trained animals compared to controls which had received equal numbers of

shocks was observed in the hippocampus immediately following training, which had declined to control levels after 70 mins, and which was followed by a four-fold increase in the stable bound (vesicular) ACh in this brain area, and followed later by a smaller increase in labile bound ACh. Smaller increases occurred in the visual cortex, and there were no changes in the corpus striatum. These changes were taken as indications of different functional states of cholinergic terminals at different times during acquisition and consolidation of learned behaviour.

Using the same training situation, intraperitoneal injection of a cholinolytic prior to training impaired the acquisition and retention of this task, but improved the retention when injected immediately after training, and this was to some extent dose dependent. Intrahippocampal injection of atropine prolonged retention when injected before or immediately after training, but not 24 h later. Acetylcholine synthesis was blocked by application of naphthylvinyl-pyridine prior to training, and this prevented the increases in the free and vesicular ACh, while injection post-trial blocked the vesicular increase, suggesting that newly synthesised ACh was needed. This inhibitor also prolonged the retention of the discrimination if administered 2 h before training.

On the basis of these results, the authors concluded that during acquisition of this task, ACh accumulates in synaptosomes of the septo-hippocampal fibres and which was released following the training, inducing an increased postsynaptic activation facilitating the extinction of the habit. While these experiments are suggestive, the role of the hippocampus in memory formation in animals is far from clear, although this

structure has certainly been implicated in human memory pathology (Weiskrantz, 1977, 1978). It is also necessary to distinguish between changes in the metabolism of the neurotransmitter which are a consequence of functional changes induced by pharmacological intervention and those which cause a functional change, and this is not always clear.

It may also be noted that in man anticholinergic glycolate esters such as quinuclidinyl benzilate (QNB) can cause complete amnesia (Abood, 1968) 1-8 hours after I.M. injection, and a pharmacological study on humans indicates that the cholinergic system is involved in age related memory degeneration (Drachman and Leavitt, 1974). Further, in an examination of cortical biopsies removed at craniotomy from patients with chronic memory defects associated with nerve cell loss in Alzheimer's disease, a reduction of over 50% in CAT activity has been detected while GAD activity remained unaltered compared to controls (Spillane *et al.*, 1977).

There is some evidence then *inter alia* implicating the functional involvement of cholinergic synapses at the neocortex with the consolidation or recall of experience. It is not, of course, excluded that functionally significant changes may be occurring at synapses associated with other transmitter systems, either simultaneously or with different temporal or other characteristics. This evidence has been discussed as a background to the experiments presented in the remainder of this thesis, and a further discussion in the light of the results is presented in Chapter 8.

CHAPTER FOUR

BIOCHEMICAL ASSAY PROCEDURES

For the experiments described in the remainder of this thesis, the levels of the enzymes acetylcholinesterase (AChE) and cholineacetyltransferase (CAT) and ^{3}H QNB specific binding to the muscarinic cholinergic receptor were determined in homogenates of visual and motor cortex by methods established according to the results of the following preliminary procedures.

Acetylcholinesterase levels were measured by the spectrophotometric method of Ellman et al. (1961), which measures the enzyme activity by following the increase of yellow colour produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. Assay conditions were established according to the results of the procedures illustrated in Figs. 4.1, 4.2, 4.3 and 4.4. Fig. 4.1 illustrates the assay to be linear with the adopted conditions with respect to increasing amounts of tissue over the range determined (0.1-1.8 mg). Each point represents the mean ± S.E.M. from four aliquots of the same homogenate. Amounts of tissue in the range 0.3 to 0.4 mg were routinely used, corresponding to a 20 µl aliquot of the homogenate. Fig. 4.2 illustrates the effects of differing substrate (acetylthiocholine iodide, Sigma) concentrations with this amount of tissue. Each point represents the mean±S.E.M. of 4 determinations. No significant increase in measured AChE activity was observed with increasing concentrations added above 0.075 M and this concentration - identical to that used in the original Ellman paper - was adopted. The concentrations of dithiobisnitrobenzoic acid (DTNB) and buffer (0.1 M, pH = 8.0) were also used as reported in the original paper. Finally, the

assay was checked for temporal linearity and temperature sensitivity. Fig. 4.3 illustrates the results obtained with incubation times of from 5 to 80 minutes. Each point represents the mean ± S.E.M. of 4 determinations and reveals the reaction to be essentially linear over the initial 20-30 minutes of incubation time. Fig. 4.4 illustrates the means ± S.E.M. of 6 determinations, and reveals maximal AChE activity to occur at around 40° C. The final assay conditions adopted and used throughout all subsequent experiments were as described below.

Duplicate 20 µl aliquots of the homogenate were taken and each added to 2.9 ml of a solution containing potassium phosphate buffer 0.1 M, pH = 8.0, 5:5-dithiobis-2-nitrobenzoic acid 0.34 mM, and sodium bicarbonate 0.6 mM. 10 µl of tetraisopropylpyrophosphoramide 100 µM (Koch-Light) were added to inhibit non-specific cholinesterase (butyrylcholinesterase activity). 20 µl of acetylthiocholineiodide 0.075 M (Sigma) were added and the tubes immediately incubated for 20 minutes at 37° C in a shaking water bath. The samples were put on ice and read off the ice having attained a temperature of approximately 5° C, against a blank containing reaction mixture only and with reference to a standard tissue control, on a Pye SP6 -500 spectrophotometer.

Two further observations have validated these assay conditions. The average value for AChE activity found throughout these experiments and reported here and in published work (Wood and Rose, 1979) is similar to that previously reported in the literature for rat cortex (see Table 4.1) with the use of the Ellman method.

Legend to Figures 4.1 to 4.4

Results of acetylcholinesterase assay procedures. Each point represents the mean ± S.E.M. of 4 (Figs. 4.1, 4.2, 4.3) or 6 (Fig. 4.4) determinations with methods as described in the text.

Fig. 4.1: Tissue concentration

Fig. 4.2: Acetylthiocholineiodide molarity

Fig. 4.3: Incubation time

Fig. 4.4: Incubation temperature

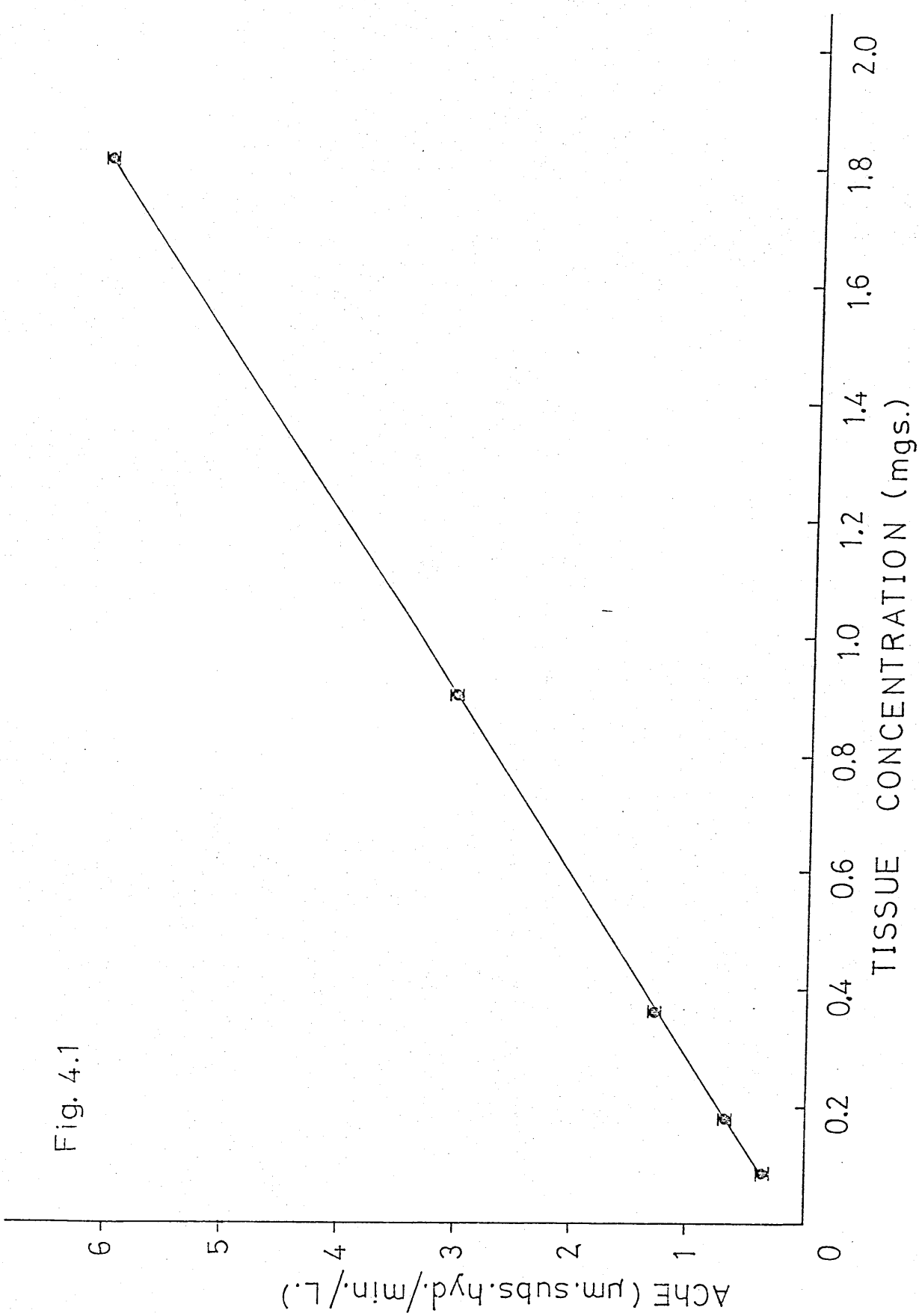

AChE (µm.subs.hyd./min./L.)
0
1
2
3
4
5
6
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
TISSUE CONCENTRATION (mgs.)

Fig. 4.1

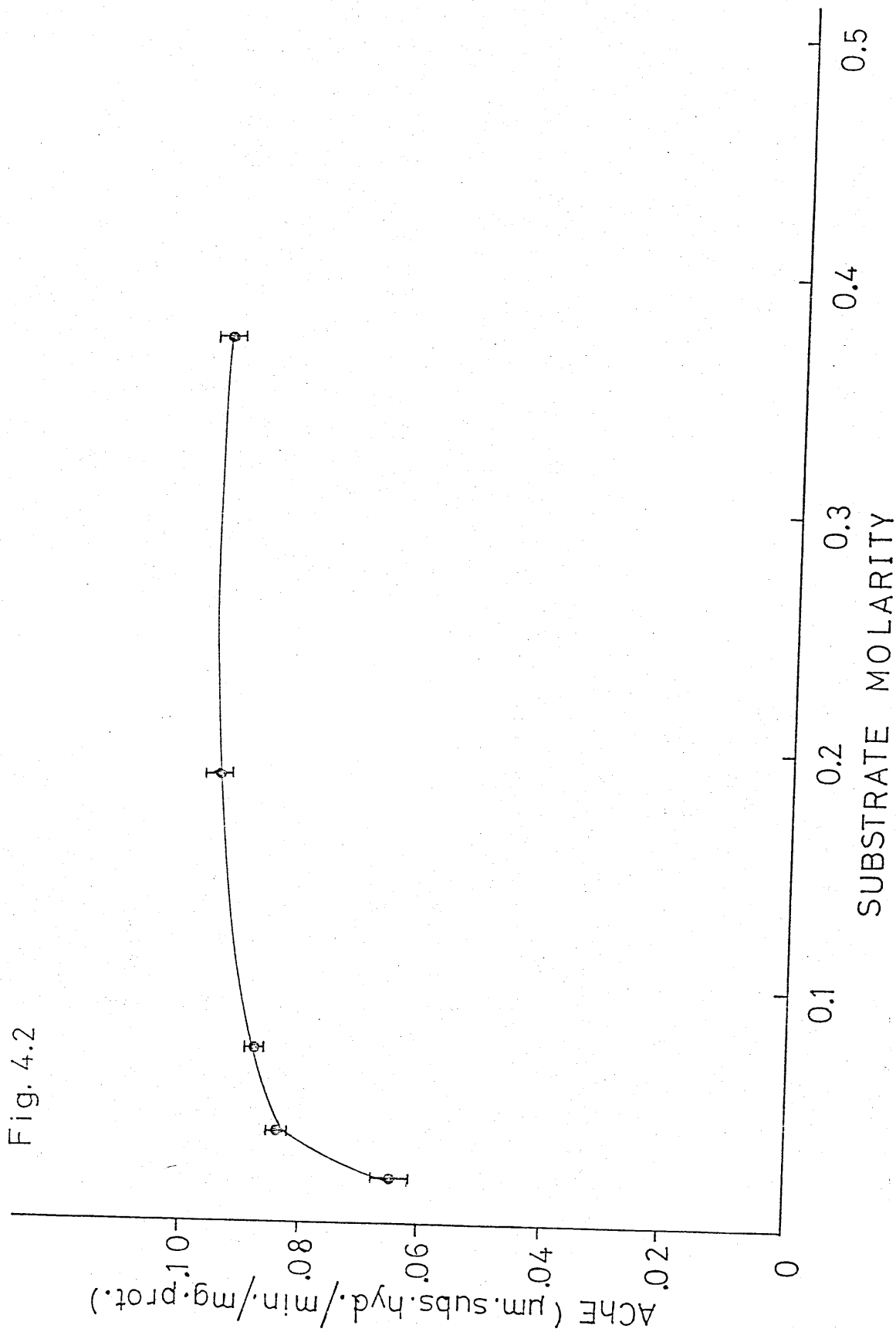

ACHE (µm.subs.hyd./min./mg.prot.)
0
.02
.04
.06
.08
.10
0.1
0.2
0.3
0.4
0.5
SUBSTRATE MOLARITY

Fig. 4.2

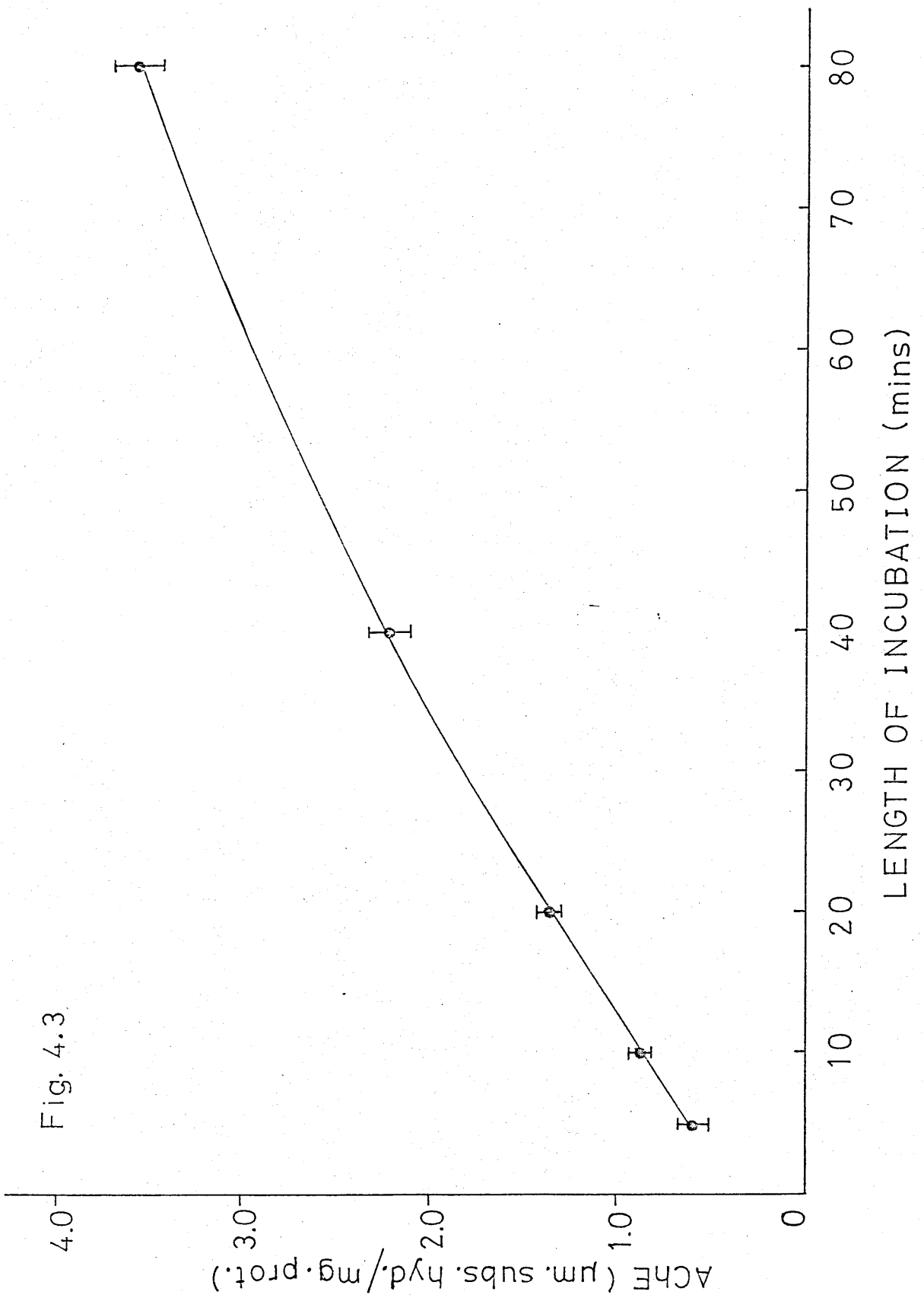

ACHE (μm. subs. hyd./mg. prot.)
4.0
3.0
2.0
1.0
0
10
20
30
40
50
60
70
80
LENGTH OF INCUBATION (mins)

Fig. 4.3

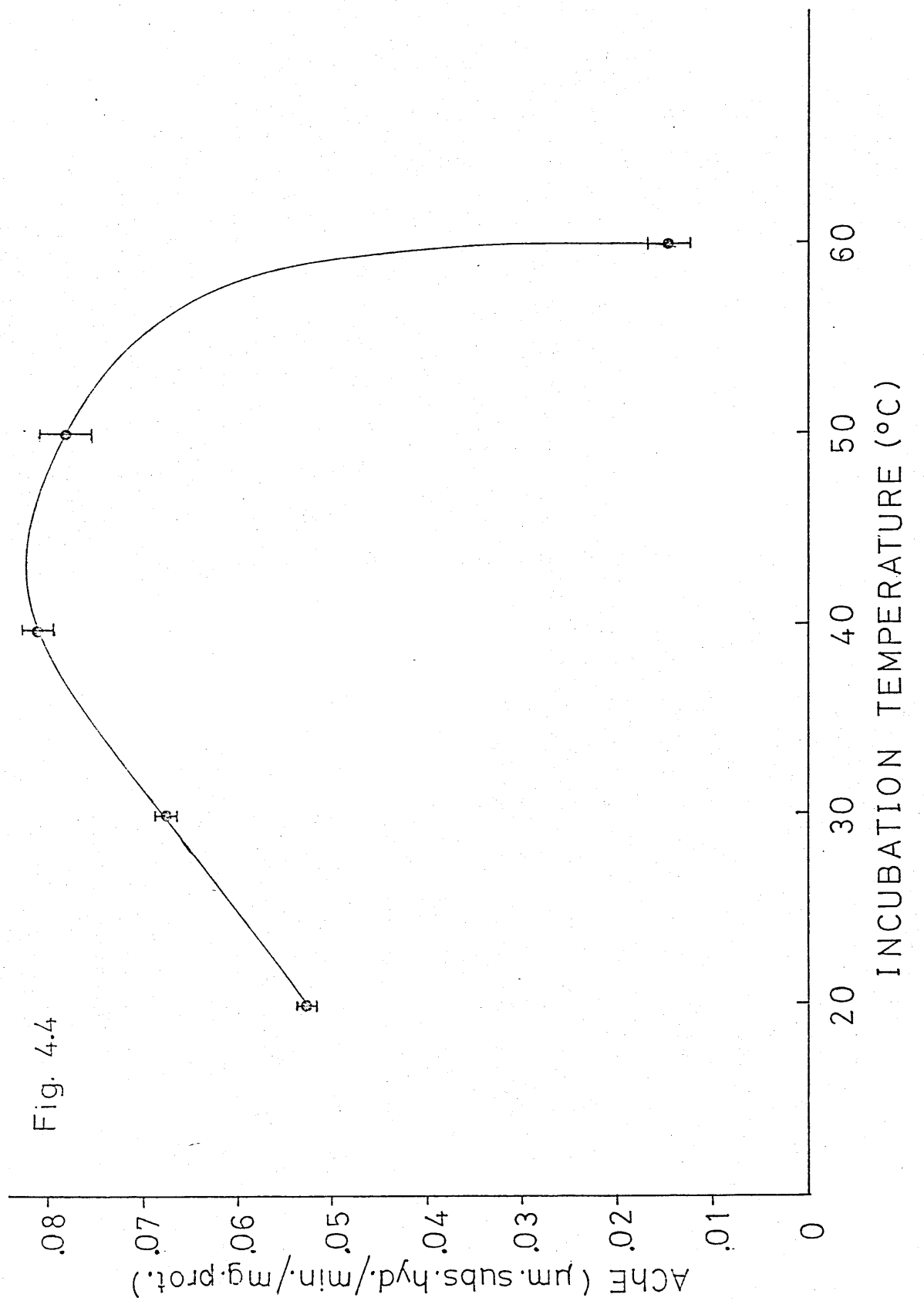

AChE (µm.subs.hyd./min./mg.prot.)
.08
.07
.06
.05
.04
.03
.02
.01
0
20
30
40
50
60
INCUBATION TEMPERATURE (°C)

Fig. 4.4

TABLE 4.1

Comparison of absolute reported values of acetylcholinesterase activity in rat brain regions

Source	Conditions	Reported average value	Average activity converted to µm substrate hydrolysed /min/mg protein
Quay et al., (1971)	dorsal cortex decapitation S_1 strain	6.3 nm/min/mg tissue	0.063
Wood and Rose(1979a)	visual and motor cortex 7 week old stunning Wistar CFHB	0.085 µm/min/mg protein	0.085
McKinney (1970)	whole brain 7-10 week old stunning Sprague Dawley	98 M/min/mg tissue $\times 10^{10}$	0.098
Ellman et al. (1961)	whole brain Sprague Dawley	10.3 M/l/min/g tissue $\times 10^{-6}$	0.103
Moudgil et al. (1973)	cerebral hemispheres 7 week old cervical dislocation Wistar	150 units/mg protein $\times 10^{3}$	0.150

Acetylcholinesterase activity has been expressed throughout as µmoles of substrate hydrolysed per minute per milligram of protein according to the following formula (adapted from Ellman et al., 1961):-

$$R = \frac{\Delta A}{1.36 \times 10^4} \times \frac{1}{(20/2950)\ C_0} \times 10^3$$

where R = the rate in µmoles substrate hydrolysed per min per mg protein; ΔA = the change in absorbance units per minute; C_0 = the original concentration of protein in mg per ml.

1.36×10^4 = a constant of the extinction coefficient of the yellow anion

20/2950 = the proportion of tissue aliquot to total volume.

Furthermore, the mean agreement of 50 randomly selected duplicate determinations of acetylcholinesterase activity from the experimental data was revealed to be < 6%, a variation which could largely be accounted for by pipetting error.

Cholineacetyltransferase (CAT) activity was determined from the tissue homogenates which had been frozen once and stored at -20° C for an average of several weeks. Preliminary experiments had shown this freezing and thawing procedure to have no detectable effect on measured CAT activity (see Table 4.2).

TABLE 4.2

The effects of one freezing and thawing on CAT activity for four determinations in each of two rat cortex homogenates. Results are expressed as DPM/mg protein/h and arranged in decreasing order.

	SAMPLE 1		SAMPLE 2	
	Fresh tissue	Frozen	Fresh tissue	Frozen
	165657	159091	156566	147475
	156061	157576	140404	140657
	145455	156328	152526	132324
	140908	134343	126768	122223
Means:	152020	151835	144066	135670

Cholinacetyltransferase activity was measured by a slight modification of the radiochemical method of Fonnum (1975), which is a simplified method of the Fonnum (1969) procedure whereby labelled ACh is extracted by liquid cation exchange using sodium tetraphenylboron in ethyl butyl ketone. With the present method, the scintillation cocktail is used as the extraction solvent and is carried out directly in the

scintillation vial, ^{14}C labelled ACh subsequently being determined in a biphasic aqueous/toluene scintillation mixture. In the published paper, Fonnum used 2 µl of enzyme solution in a microtube and 5 µl of substrate mixture, which were then incubated and washed into a scintillation vial. The present modification scales up these quantities by a factor of 10, to eliminate any inaccuracies which might be introduced with the microtechniques, the new procedure enabling the incubation to proceed directly in the scintillation vial. The assay conditions were established according to the results of the procedures illustrated in Figs. 4.5 and 4.6. Figure 4.5 reveals the assay to be linear with the adopted conditions with respect to increasing amounts of tissue over the range 0.1 to 0.4 mg. Each point represents the mean ± S.E.M. from four aliquots of the same homogenate. This graph also indicates the high agreement obtained between aliquots containing the same amount of protein.

Amounts of tissue in the range 0.15-0.18 mg were subsequently used for the experiments, corresponding to a 20 µl aliquot of the standard homogenate rehomogenized 1:1 with EDTA (see below). Figure 4.6 illustrates the effects of different incubation substrate mixture volumes. Each point represents the mean ± S.E.M. of 4 determinations. It indicates no significant further increase in measured CAT activity above 50 µl, which quantity was subsequently adopted. This proportion of 20 µl of enzyme solution to 50 µl incubation mixture represents the same proportion as that originally reported in the Fonnum paper (1975). Finally, the mean agreement of 50 randomly selected duplicates used throughout this series of experiments was < 6.5%. Incubation time and

Legend to Figures 4.5 and 4.6

Results of cholineacetyltransferase assay procedures. Each point represents the mean ± S.E.M. of 4 determinations with methods as described in the text.

Fig. 4.5: Tissue concentration

Fig. 4.6: Volume of substrate mixture.

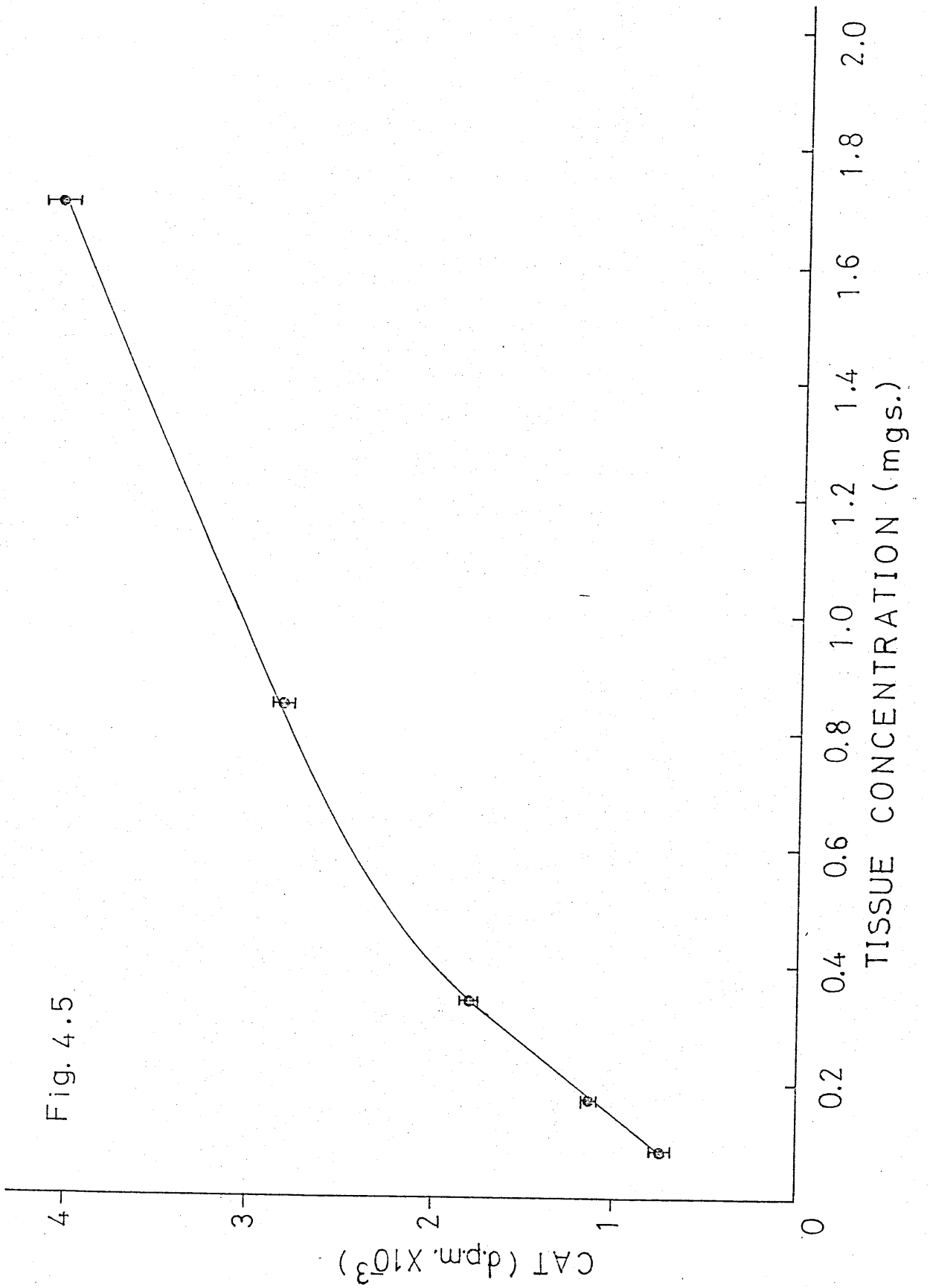

CAT (d.p.m. X10⁻³)
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
TISSUE CONCENTRATION (mgs.)

Fig. 4.5

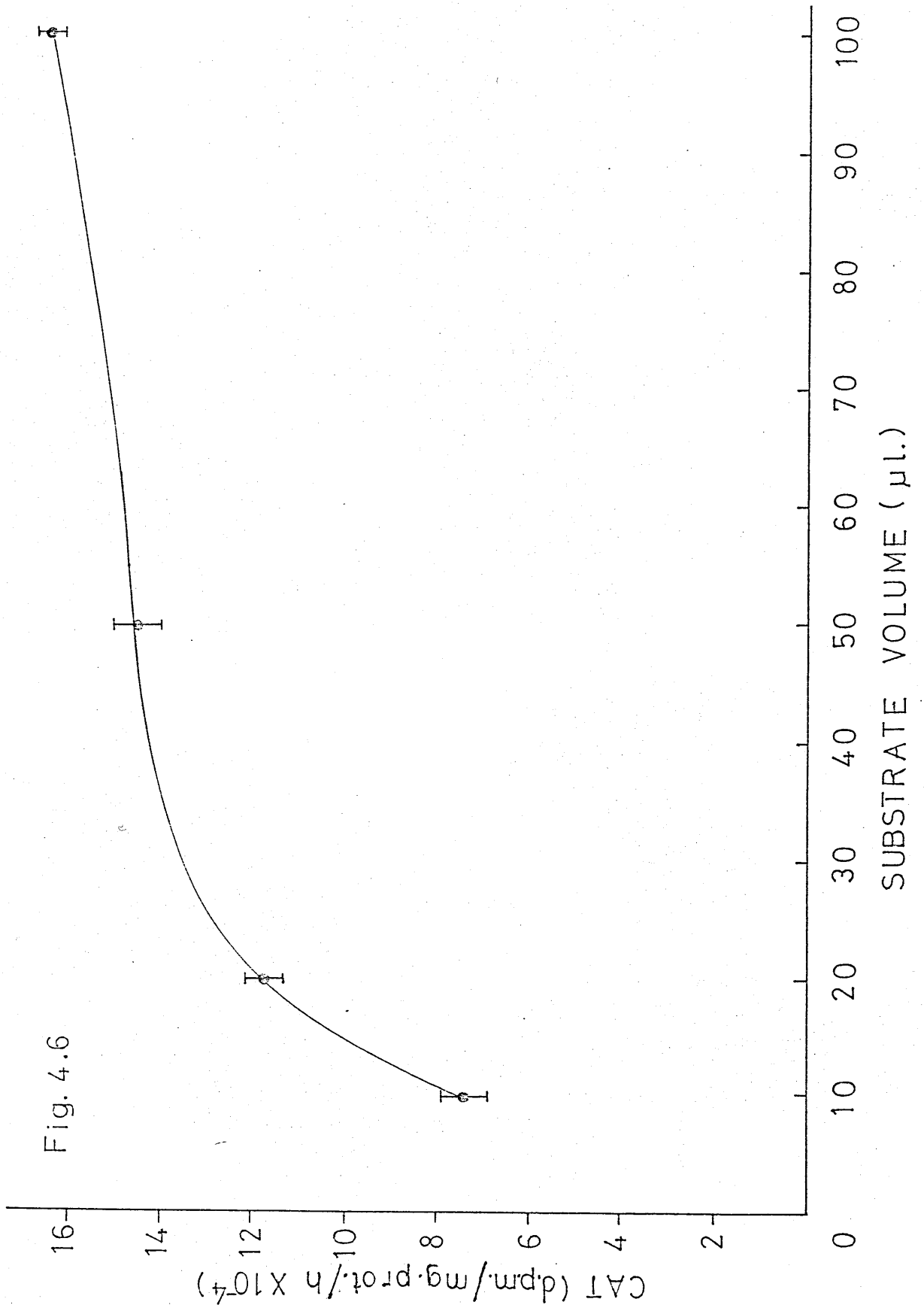

CAT (d.p.m./mg.prot./h X10⁻⁴)
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
90
100
SUBSTRATE VOLUME (µl.)

Fig. 4.6

temperature were used as originally reported in the Fonnum paper. Further experiments confirmed the assay to be linear over the initial 15-20 minutes incubation. The final assay conditions adopted throughout all subsequent experiments were as described below.

Frozen homogenates, prepared as described in Chapter 5, were thawed and thoroughly remixed. 0.5 ml aliquots of each homogenate were removed and thoroughly mixed with an equal volume of EDTA 20 mM, pH 7.4, containing 1% Triton X-100, to activate the homogenates and ensure total release of enzyme activity. Duplicate 20 µl aliquots of these homogenates were taken and added to 50 µl of incubation mixture in a scintillation vial. The incubation mixture contained sodium phosphate buffer 50 mM, pH 7.4, choline bromide 8 mM, EDTA 20 mM, physostigmine 0.1 mM and ^{14}C acetyl-CoA 0.2 mM. The ^{14}C-acetyl-CoA (Radiochemical Centre, Amersham: equivalent to c. 110,000 dpm per vial) was diluted with the unlabelled compound (Sigma). The vials were incubated for 15 min at 37° C in a shaking water bath. Immediately on removal from heat, 5.0 ml of ice cold sodium phosphate buffer 50 mM, pH 7.4, were added, followed by 2.0 ml of extractant/scintillation cocktail containing 5.0 g/l PPO, 0.833 g/l sodium tetraphenylboron and acetonitrile and toluene in a proportion 1:5. To extract the ACh into the toluene phase, the vials were regularly and slowly inverted for one minute. Samples were counted at 3% error on a Beckman LS-150 or LS-250 liquid scintillation counter. CAT activity has been expressed throughout as disintegrations/minute/milligram protein/hour.

^{3}H quinuclidinylbenzilate (QNB) binding to the muscarinic acetylcholine receptor (mAChr) was estimated by a modification of the method of Yamamura and Snyder (1974). Binding sites with

high affinity and specificity for ^{3}H QNB have been shown to be present in rat brain homogenate, which binding is displaced by muscarinic but not nicotinic ligands. All assays were performed on tissue homogenates, prepared as described, which had been frozen and thawed twice having been stored at -20° C. Rose and Stewart (1978) have established that freezing and thawing up to three times has no detectable effect on measured ^{3}H QNB binding. The assay conditions were established according to the results of the procedures illustrated in Figs. 4.7, 4.8 and 4.9. Figure 4.7 illustrates the results of varying the concentration of a tissue aliquot by dilution. Each point represents the mean ± S.E.M. of 6 determinations (assay conditions were as described below). The graph indicates the measured binding to increase in direct proportion to increasing amounts of tissue in the homogenate, using the adopted conditions, over the range 0.25-2.0 mg above which saturation occurs, and amounts of tissue within this linear range were subsequently used. ^{3}H QNB binding in the presence of atropine remained at a constantly low level of approximately 0.02 pmoles.

Fig. 4.8 illustrates the effects of varying the concentration of ^{3}H QNB over the range 0.5 nM to 12.0 nM in the presence and absence of atropine. Specific binding was defined as the total binding minus the binding in the presence of the atropine. Under these conditions, the ratio of specific to non-specific binding was found to be at the various QNB concentrations 0.5 nM = 11.1:1, 1.0 nm = 11.4:1, 3.0 nM = 20.1:1, 6 nM = 13.3:1 and 12 nM = 10.5:1, providing an average figure of 13.3:1. ^{3}H QNB binding in the presence of

Legend to Figures 4.7 to 4.9

Results of ^{3}H QNB binding assay procedures. Each point represents the mean ± S.E.M. of 6 (Figs. 4.7 and 4.8) or 3 (Fig. 4.9) determinations with methods as described in the text. Round points - binding in the presence of 10 µM atropine. Cross points - binding in the absence of atropine.

Fig. 4.7: Tissue concentration

Fig. 4.8: ^{3}H QNB molarity

Fig. 4.9: Atropine molarity

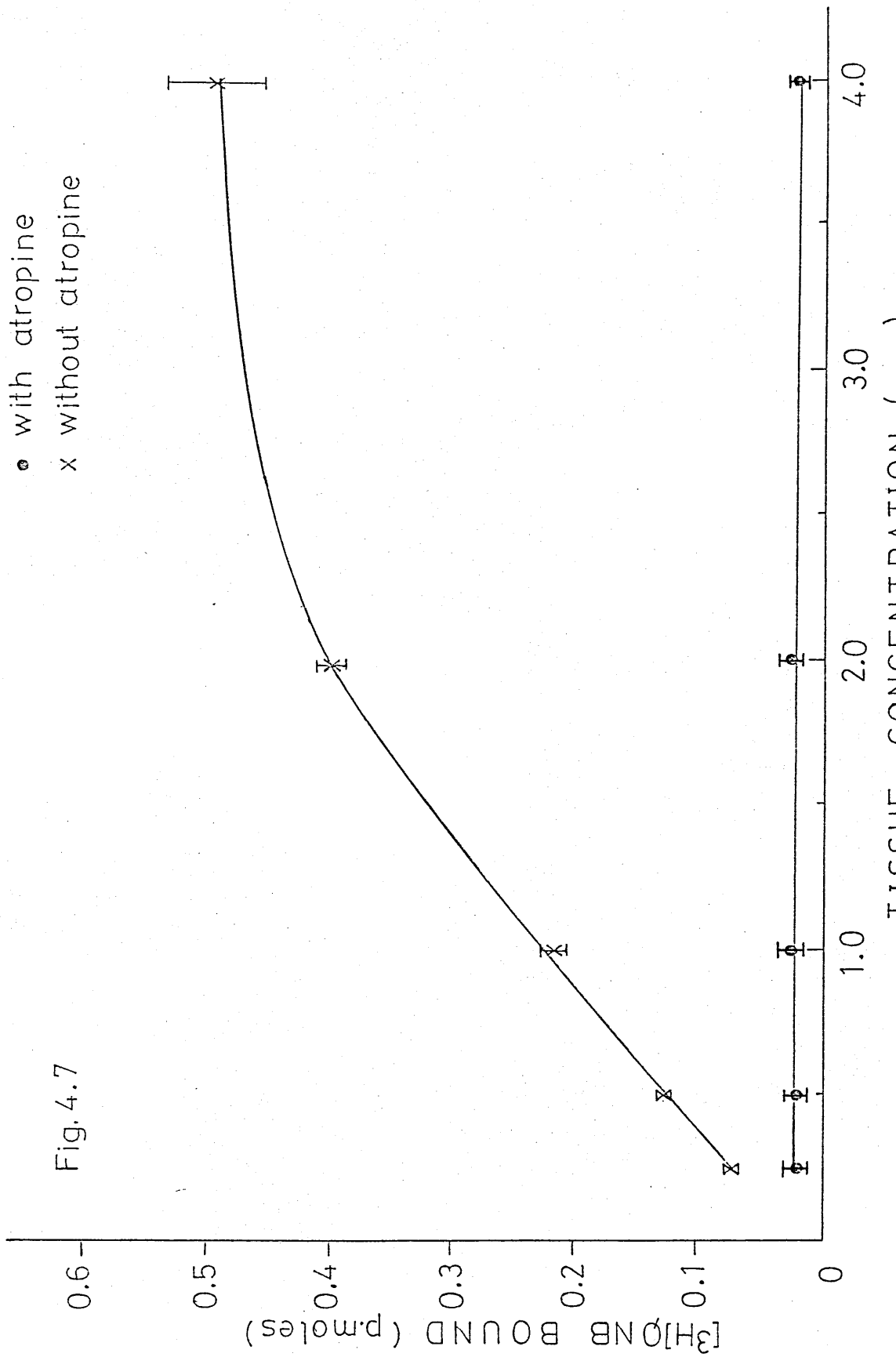

• with atropine
x without atropine
[3H]QNB BOUND (p.moles)
0.6
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
TISSUE CONCENTRATION (mg.)

Fig. 4.7

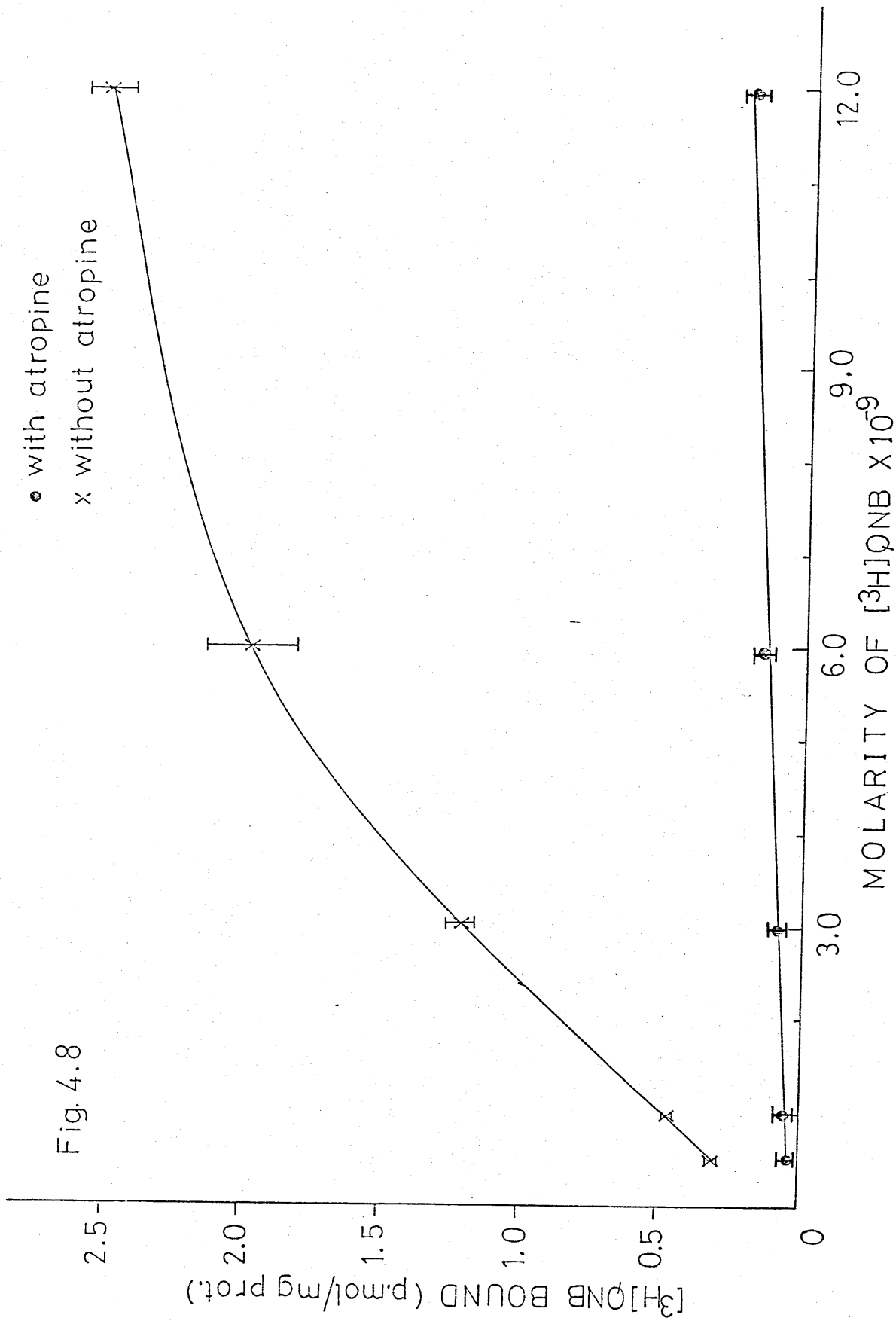

• with atropine
x without atropine
[3H]QNB BOUND (p.mol/mg prot.)
2.5
2.0
1.5
1.0
0.5
0
3.0
6.0
9.0
12.0
MOLARITY OF [3H]QNB X10-9

Fig. 4.8

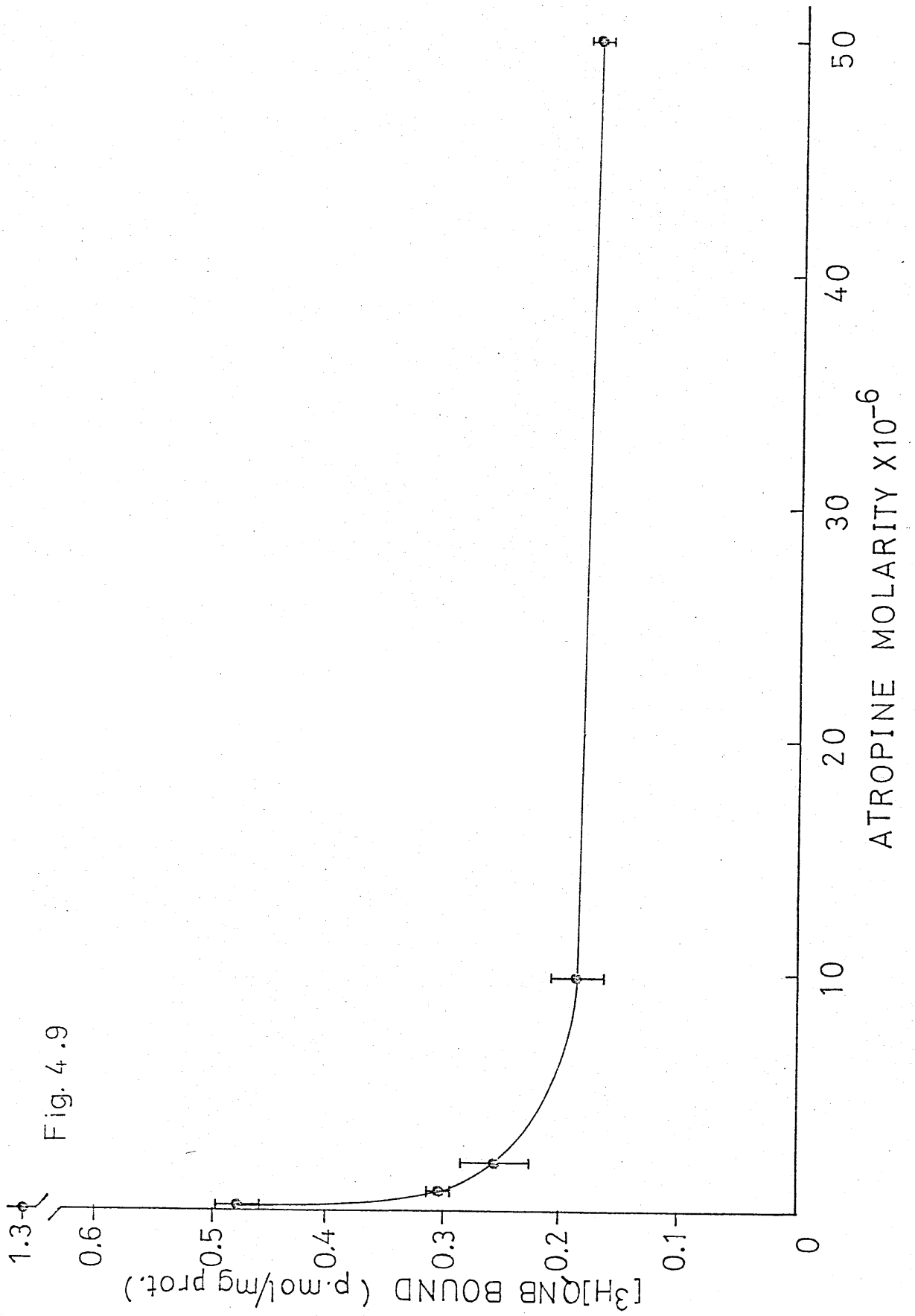

[3H]QNB BOUND (p.mol/mg prot.)
1.3
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
ATROPINE MOLARITY X10-6

Fig. 4.9

atropine only slowly increases with higher concentrations of ^{3}H QNB, indicating the much higher affinity at the concentration used (10 µM) of the classical antagonist for the muscarinic receptor. These binding figures can be compared with those reported by Yamamura and Snyder (1974) who found the ratio of specific to non-specific binding to be in the range 8-10:1 using 100 µM oxotremorine or cold QNB in place of atropine. The concentration of 6 nM ^{3}H QNB was chosen for further experiments. Fig. 4.9 illustrates the effects of altering atropine concentration using the 6 nM concentration of ^{3}H QNB. Each point represents the mean ± S.E.M. of triplicates. Binding in the absence of atropine was found to be approximately 1.3 pmole bound/mg protein, which had fallen to 37% of this value with an atropine concentraion of 100 nM. Increasing the concentration to 10 µM provided virtually maximal blocking of ^{3}H QNB binding, whilst a five-fold further increase of atropine concentration provided only a further 7% decrease in ^{3}H QNB binding. Accordingly a concentration of 10 µM atropine sulphate was chosen. The final assay procedure used was based on the Rose and Stewart (1978) modification of the Yamamura and Snyder (1974) method and was as follows.

Triplicate 100 µl aliquots of thawed and remixed homogenates (containing approximately 150-180 µg protein) were taken and added to a reaction mixture containing 6 nM ^{3}H QNB (Radiochemical Centre, Amersham), specific radioactivity = 8.4 Ci/mmol and sodium-potassium phosphate buffer 0.05 M, pH = 7.4. A further three 100 µl aliquots of the homogenate were taken and added to the incubation mixture containing 10 µM atropine sulphate (Sigma). The final volume in each case was 2.0 ml. These sextuplicates were incubated for 1 h at

25° C in a shaking water bath. Following incubation, the contents of the tubes were poured through 2.5 cm GF/B Whatman filter discs on a Millipore manifold under suction. The contents of the tubes were washed twice with 2.0 ml ice-cold Na-K phosphate buffer, and the filters washed a further three times with 2.0 ml of the buffer. Assays were generally performed with 30 discs at a time which, following washing, were dried for approximately 2 h at 50° C. These were put into scintillation vials with a toluene-methoxyethanol (50:50) scintillant containing 6 g/l PPO and 1% Triton X-100, and counted on a Beckman LS 250 at 3% counting error. The mean deviation of the samples from the means of each of 30 randomly selected triplicates from the experimental data was < 8% (without atropine) and < 17% (with atropine).

Duplicate 100 µl aliquots of homogenate were further taken for protein estimation by the method of Lowry et al. (1951) in a total volume of 4.5 ml and read against a distilled water blank using a BSA standard (1 mg/ml) at 500 nm on a Pye SP6-500 spectrophotometer. The mean agreement of 50 randomly selected duplicate determinations of protein from the experimental data was found to be < 5%.

CHAPTER FIVE

CIRCADIAN VARIATIONS AND ACETYLCHOLINESTERASE

The increase of up to 30% in acetylcholinesterase (AChE) activity which had been observed in the visual cortex of three hour light exposed animals (Sinha and Rose, 1976) was chosen as the biochemical marker for the experiments described in this chapter, as a reliable and readily reproduced index of possible altered neuronal activity, presumably an index of ACh turnover, although the enzyme is not so specific a cholinergic synaptic marker as cholineacetyltransferase. One explanation which was advanced in Chapter 2 to account for the failure to detect any increase of ^{3}H lysine incorporation in 21 day old light exposed animals was that exposure to the new stimulus may have occurred too late in the day, and that a circadian fluctuation in biochemical response may have been present. This hypothesis was tested directly in the experiments described in this chapter.

Materials and Methods

Animals

The animal rearing procedure was exactly as that described in Chapter 2. Animals were used at 50 ± 5 (range) days of age at which age they weighed, in these experiments, 200 ± 30 (range) grams.

Experimental Procedure

All experiments took place within a separate behaviour room, thermostatically controlled to a temperature of approximately 20° C, and acoustically quiet. Approximately one half of the dark reared animals were individually placed in transparent plastic cages measuring 42 cm x 27 cm x 22 cm (light exposed, L). Exposed animals were permitted auditory but not

visual contact with each other, and no food or water (see Plate 2). The other dark reared animals were placed in similar individual cages inside dark boxes, identical to that illustrated in Plate 1, and placed in close proximity to the light exposed animals. Normal animals were also placed in individual transparent plastic cages on the same bench. The light exposed and normal animals were exposed to overhead illumination from a single tungsten light at a distance of approximately 1.5 m (luminous flux approx. = 200-220 lumens/m^2 at the bench). Following three hour periods of exposure to their respective conditions between the hours of 06.00 and 21.00, animals were killed by a sharp blow to the back of the neck, and visual and motor cortices were dissected freehand and placed on ice as described in Chapter 2. All dark control animals were killed quickly in very dim illumination.

Biochemical Procedure

Left and right halves of each brain region were pooled, separately for each region, providing approximately 80-100 mg (wet weight) of tissue. Further procedures were then either performed immediately or these cortical slabs were stored at -20° C for later assay. Preliminary experiments indicated the freezing to have no detectable effect on subsequently measured acetylcholinesterase levels. The brain areas were homogenized with an MSE top-drive macerator for 45 s at 14,000 RPM in 5.0 ml of double distilled water and placed on ice. Acetylcholinesterase activity and protein concentrations were determined by the methods previously described (Chapter 4).

Results and Discussion

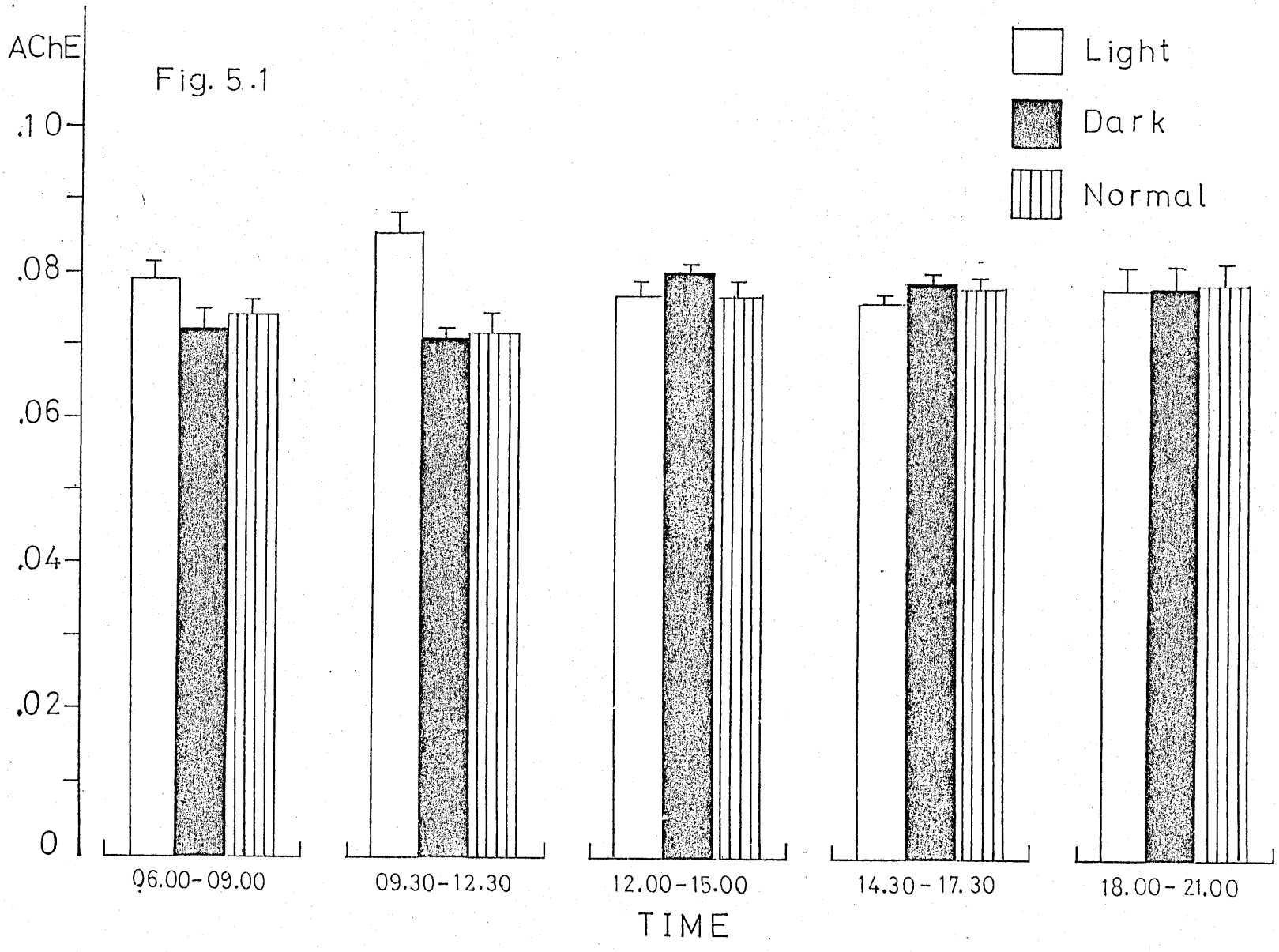
Figures 5.1 and 5.2 illustrate the results of five separate series of experiments using three hour exposure periods between the times of 06.00 and 21.00. Fig. 5.1 represents the mean AChE levels determined in the visual cortex ± S.E.M., and Fig. 5.2 represents the mean AChE levels in the motor cortex ± S.E.M. As the mean levels of AChE determined in individual litters/experiments varied up to ± 25% (range) from the mean of the total series of experiments, the means of individual litters were standardized to the common figure in order to eliminate this unwanted variation.

Acetylcholinesterase activity in the motor cortex is some 10% higher than that in the visual areas, and this essentially replicates the observations of Sinha and Rose (1976) and Quay et al. (1971). There are no significant differences at any time between the normally reared animals and that of their dark reared and unexposed littermates in either brain area, and this replicates the observations of Sinha and Rose (1976) and has subsequently been confirmed by Rose and Stewart (1978) and Bigl and Biesold (1978). This result then provides no evidence that visual deprivation depresses activity in unspecific cholinergic systems which may facilitate transmission within the visual system.

These two graphs also illustrate the effects of light exposure at the various time periods. The increase in visual cortex activity of 21% when exposure commenced at 09.30 is significant ($t = 5.12$, D.F. = 20, $p < 0.001$). The small elevation in visual cortex enzyme of 10% when exposure commenced at 06.00 is not significant ($t = 1.99$, D.F. = 6),

Legend to Figures 5.1 and 5.2

The effect of 3 h light exposure on acetylcholinesterase levels in visual cortex (Fig. 5.1) and motor cortex (Fig. 5.2) of 50 day old dark reared and light exposed (white bars), dark reared and unexposed (dark bars) and normally reared littermate rats (striped bars) at the times shown. Enzyme activity is expressed as µmoles of substrate hydrolysed/min/mg protein. Each bar represents the mean ± S.E.M. of animals in each condition. The total 'N' at each time period is as follows: 06.00-09.00 = 12, 09.30-12.30 = 32, 12.00-15.00 = 31, 14.30-17.30 = 15, 18.00-21.00 = 12. Visual cortex L/D = 1.21 at 09.30-12.30 ($t = 5.12$, $p < 0.001$).


AChE
Fig. 5.1
.10
.08
.06
.04
.02
0
Light
Dark
Normal
06.00-09.00
09.30-12.30
12.00-15.00
14.30-17.30
18.00-21.00
TIME

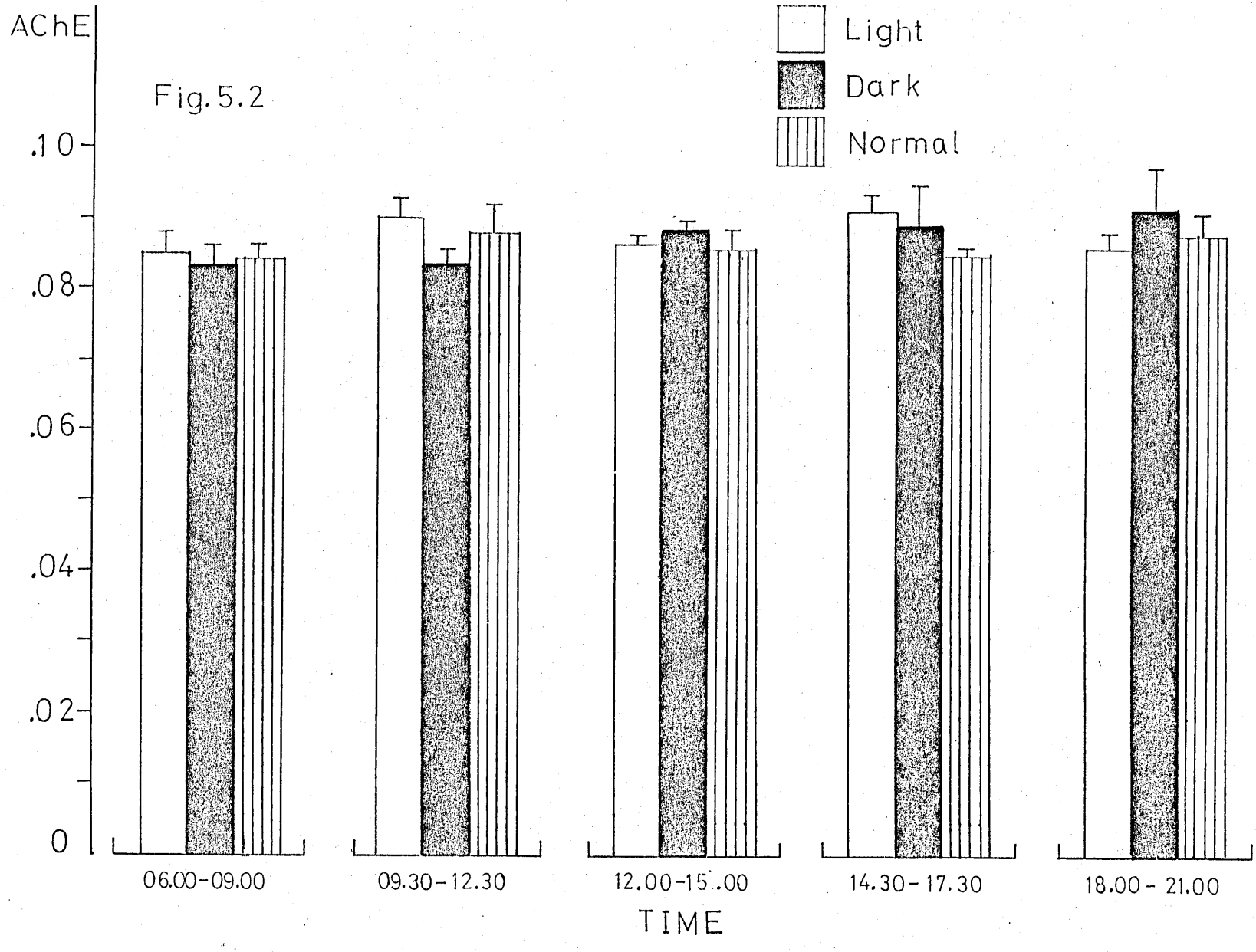

AChE
Light
Dark
Normal
.10
.08
.06
.04
.02
0
06.00-09.00
09.30-12.30
12.00-15.00
14.30-17.30
18.00-21.00
TIME

Fig. 5.2

although this strong trend might be expected to be significant with larger numbers of animals. A trend to an elevation in motor cortex enzyme activity of 8% occurred at the time (09.30-12.30) of maximal increase in visual cortex enzyme. The effects of light exposure were not significant at any other time of the day.

This increase in acetylcholinesterase levels when exposure occurred early in the day replicates the observations of Sinha and Rose (1976) and was confirmed by a later study (Stewart and Rose, 1978). The failure to detect any increase in visual cortex enzyme activity when exposure occurred later in the day (other conditions being equal) was attributed to a possible interaction with endogenous and/or exogenously controlled circadian factors. Why should this elevation be subject to circadian variation? It was noted from casual observation that animals appeared to be less active in the later afternoon, and one simple answer might be that the animals spend more time asleep during exposure. Although the animals are very alert at the commencement of the new visual experience (as might be expected!), in the latter half of the exposure period in particular, animals will spend sometimes considerable proportions of the time motionless with their eyes closed. To test this possibility of a diurnal effect, the gross levels of locomotor activity were measured in dark reared and normally reared animals over phases of the behavioural activity cycle and during light exposure, and the results of these experiments are presented later in this chapter.

The elevation in visual cortex enzyme and its diurnal interaction may also be related to general metabolic rate or

changes in blood flow. Visual stimulation is known to influence cerebral blood flow, at least in the chick (Bondy and Morelos, 1971; Bondy et al., 1974) and it may be that the effects are differential at different times of the day.

A further possibility was that endogenous levels of acetylcholinesterase may fluctuate in a regular diurnal fashion, perhaps similar to the diurnal rhythmicity in the incorporation of ^{3}H lysine into proteins of the visual and motor cortices which had earlier been described in our experimental animals (Richardson and Rose, 1971), and that this may interact with stimulus controlled influences on post-translational control or changes in the synthetic or degradative rates. In this context it is noteworthy that at least one iso-enzyme of acetylcholinesterase has been shown to have a very short turnover time, of the order of 3 h (Davis and Agranoff, 1968), and hence short term modulations of the total number of enzyme molecules present are certainly possible.

To test for this possibility of a diurnal fluctuation in AChE levels, the enzyme was measured at intervals throughout 24 hour periods in litters of dark reared and normal animals. The differences in the experimental procedure were as follows. Litters of dark reared and normal animals were chosen which contained a minimum of six (male) animals. At four hour intervals throughout the day and night, one animal was removed from a dark litter and from a normal litter, and visual and motor cortices rapidly dissected out and frozen. No other experimental procedures were performed. Each litter was therefore represented by one animal at each of

the six time points throughout the 24 hour periods. All brain samples from each litter were subsequently processed simultaneously. The biochemical procedures were identical to those described earlier.

Figures 5.3 and 5.4 illustrate the results of these experiments. Fig. 5.3 represents the results of AChE measurements determined in the visual cortex (dots) and the motor cortex (crosses) of the dark reared unexposed animals. Each point represents the mean of 9 animals ± S.E.M. Fig. 5.4 represents the same for the normally reared animals. In order to eliminate mean differences in enzyme levels between litters of animals, the results from individual litters were as before standardized to a mean common to all the litters. The results were tested for significance by the least squares averages method separately for the visual and motor cortex results, in which the null hypothesis was that no rhythm in cortical AChE was present. That is, the expected values would lie along a straight line, any deviations from this being due to chance factors. The observed values in the motor cortex of the dark reared animals differ significantly from the deviations predicted by chance (χ^2 = 15.2, D.F. = 5, $p < 0.01$) as do the values in the motor cortex of the normal animals (χ^2 = 13.7, D.F. = 5, $p < 0.02$). The values in the visual cortex in each case are not significantly different from a straight line, although from observation the rhythm in the visual cortex of the dark reared animals displays a trend to the same pattern as that in the motor cortex.

These results then indicate a certain regional specificity in the rat cortex of fluctuations in the enzyme activity, and must be considered in the light of previous

Legend to Figures 5.3 and 5.4

Circadian variation in acetylcholinesterase levels in visual cortex (dots) and motor cortex (crosses) of dark reared 50 day old (Fig. 5.3) and normally reared (Fig. 5.4) rats. Enzyme activity is expressed as µmoles of substrate hydrolysed/min/mg protein in means ± S.E.M.of N = 9 for each time point shown. Motor cortex activity over the 24 h periods differs significantly from a straight line for dark reared (χ^2 = 15.2, $p < 0.01$) and normal (χ^2 = 13.7, $p < 0.02$) animals.

Fig. 5.3

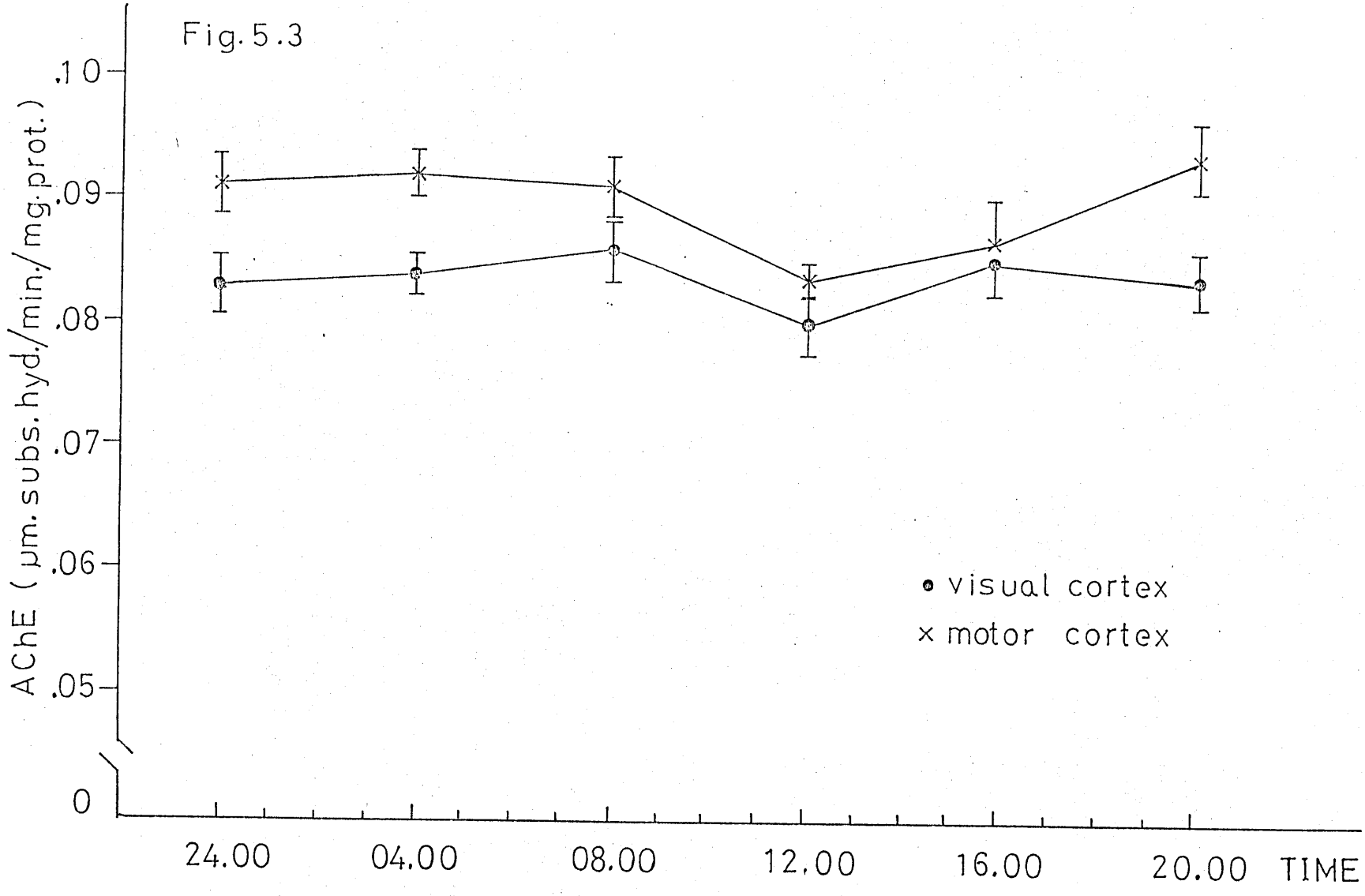
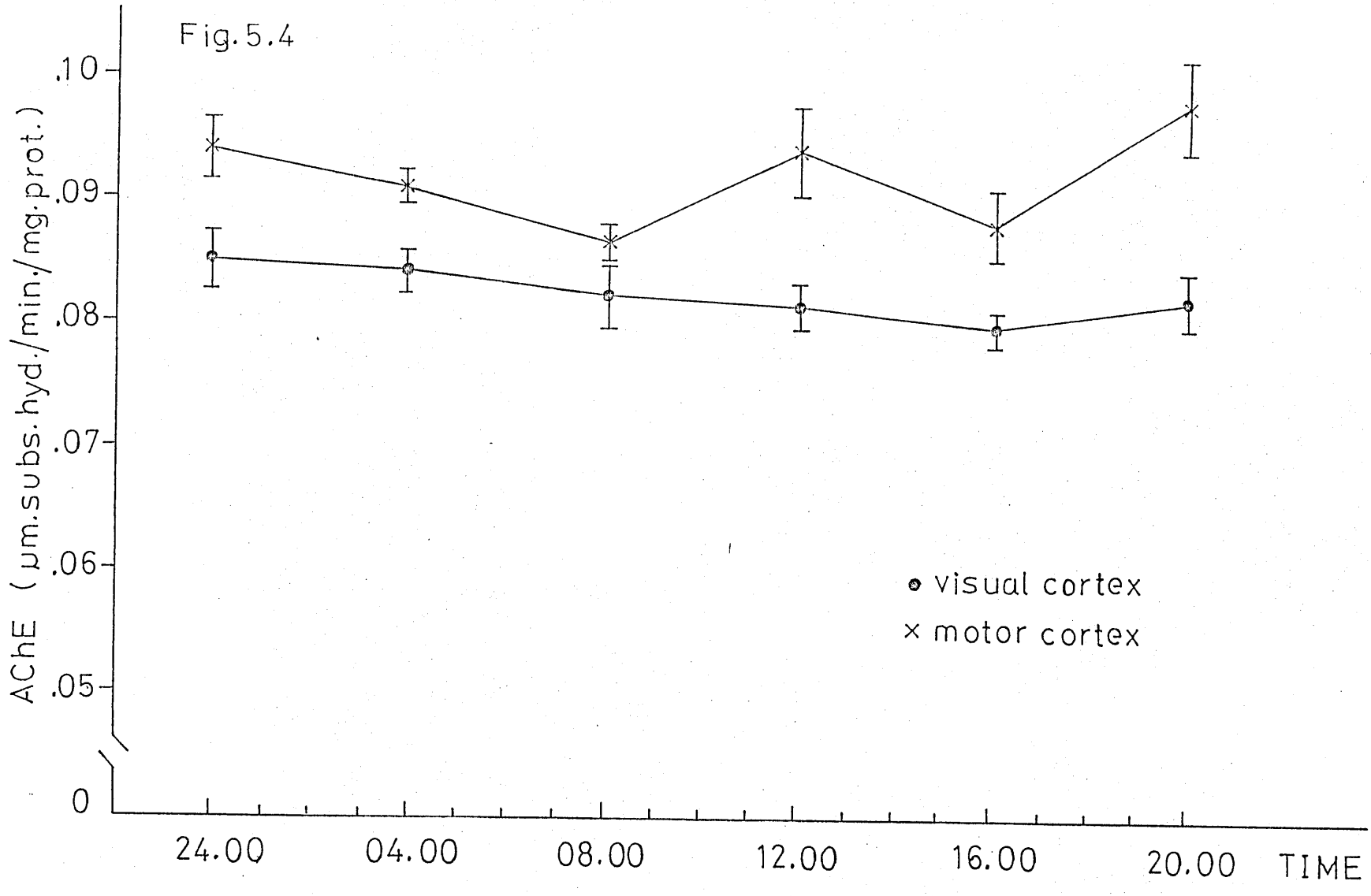

AChE (µm. subs. hyd./min./mg. prot.)
.10
.09
.08
.07
.06
.05
0
24.00
04.00
08.00
12.00
16.00
20.00
TIME
visual cortex
motor cortex

Fig. 5.4


.10
.09
.08
.07
.06
.05
0
AChE (μm.subs.hyd./min./mg.prot.)
24.00
04.00
08.00
12.00
16.00
20.00
TIME
visual cortex
motor cortex

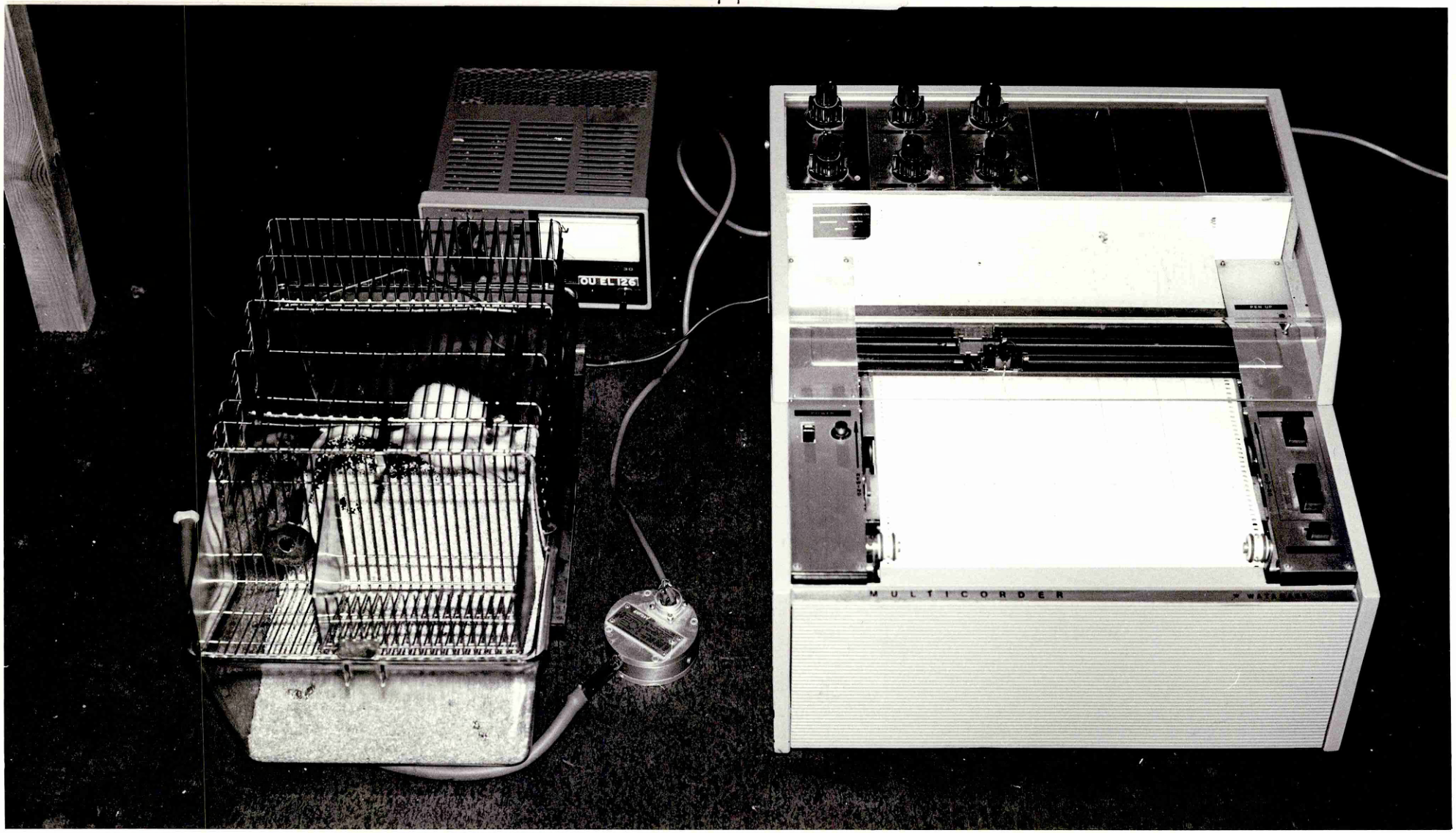
reports on diurnal variations in the cholinergic system of the rat. Moudgil and Kanungo (1973) reported a rhythm in AChE activity in normal rats which is high during the day and low at night, and which varied as a function of age of the animal. Although these results are compatible with the present ones, they are not strictly comparable as they represent activity in whole cerebral hemispheres. In addition, Saito (1971) has shown that brain acetylcholine varies, with a peak during the light and low levels during the dark period, and further that this is correlated with the daily cycle of motor activity. The evidence is contradictory, and Quay et al.(1971) found no evidence of a 24 hour rhythmicity in AChE activity in whole cerebral cortex. These results are, however, still compatible with the present ones, for if the results for the visual and motor cortices are combined in each case,the resulting rhythms are not significant. The results reported here would indicate a regional specificity in a particular functional area within the cortex. A further difference in experimental methodology is that the results of the Quay et al. study (1971) indicate caging conditions during rearing to be an important exogenous controlling factor. Their animals were housed singly in three out of four experiments, and our animals were all housed in littermate groups.

The present results also indicate a differentiation between the normally reared and dark reared animals, for although both display a significant rhythm in the motor cortex, they are not the same. The rhythm in the dark reared animals, although the amplitude is small, is clearly two-peaked,and appears to correlate with the overall loco-

motor activity cycle. The rhythm in the normal animals is more clearly four-peaked, although the large standard errors on the points at 12.00, 16.00 and 20.00 indicate considerable variability which exists between individual animals. These results may imply that with the dark box animals we are observing a simplified system, less complicated by externally mediated effects such as attention and arousal of the animals in the normal animal house environment. That animals maintained in our dark box system display a rhythmicity which is circadian and entrained to a normal 24 h cycle may not be interpreted as evidence for the endogenous driving of the clock, however, as the dark boxes are maintained in an otherwise normal animal house environment in which social, auditory and olfactory cues are not excluded from the animals' experience.

As referred to earlier in this chapter, it was decided to record the overall locomotor activity levels of the animals throughout the circadian cycle, and during light exposure at various times of the day, to attempt to correlate this with the neurochemical measures. Plate 3 illustrates the method used in these experiments. A Grass PT 5A volumetric air pressure transducer was connected by an airtight seal to a 40 cm length of rubber tubing. The whole animal cage - either inside the rearing dark box, of normal animals in the animal house or during light exposure in the behaviour room - was rested at one end on this tube. Small differences in pressure on the floor of the cage as the animals moved around resulted in slight compressions of the tube. An 8v stabilized potential difference across the transducer was thus modulated to produce an output in the millivolt range, linearly

Plate 3. The motor activity recording apparatus



dependent upon the strength of compression of the tube. This output was fed into one channel of a Watanabe multicorder 3 channel pen recorder (sensitivity = 10 mV, chart speed = 1 cm/min). Spurious noise was eliminated by only counting > 2 mm deflections on the paper. Each deflection was arbitrarily assigned as a unit of activity. The total units/hour were counted and scored for each record of each litter or animal. The sensitivity of this arrangement was such that a large whole body movement by an animal or group of animals resulted in a countable unit, while individual grooming behaviour, scratching etc. were excluded. Beyond this crude differentiation, no attempt was made to separate other components such as fast or slow actions, or movements of individual animals from that of groups, although some of this information would be retrievable from the records. Fig. 5.5 illustrates a sample portion of a recording.

The results of these experiments are illustrated in Figs. 5.6, 5.7, 5.8 and 5.9. Figure 5.6 represents the mean hourly activity ± S.E.M. of 23 to 29 measurements at each hour point, collected from the continuous records of 6 dark reared litters during dark rearing between the ages of 45-55 days each recorded continuously over a period of 4-5 days and represents the sum activity of 4-8 animals per cage. The pattern of activity in each of the dark colonies was very similar, with a similar ratio of amplitude range. The amplitude range in the litter showing the smallest amplitude was 63 activity units above the mean and 58 activity units below the mean. The corresponding figures for the litter showing the largest range were 188 activity units and 132 activity units. A large variation in

Legend to Figure 5.5

Sample of motor activity record. The chart shows the pattern obtained over a 25 minute period from two cages, each containing four 50 day old animals, recorded simultaneously with a Watanabe multicorder pen recorder, using methods as described in the text. Pen displacements > 2 mm were assigned as units of activity. Top line illustrates an active group and lower line an inactive group of animals.

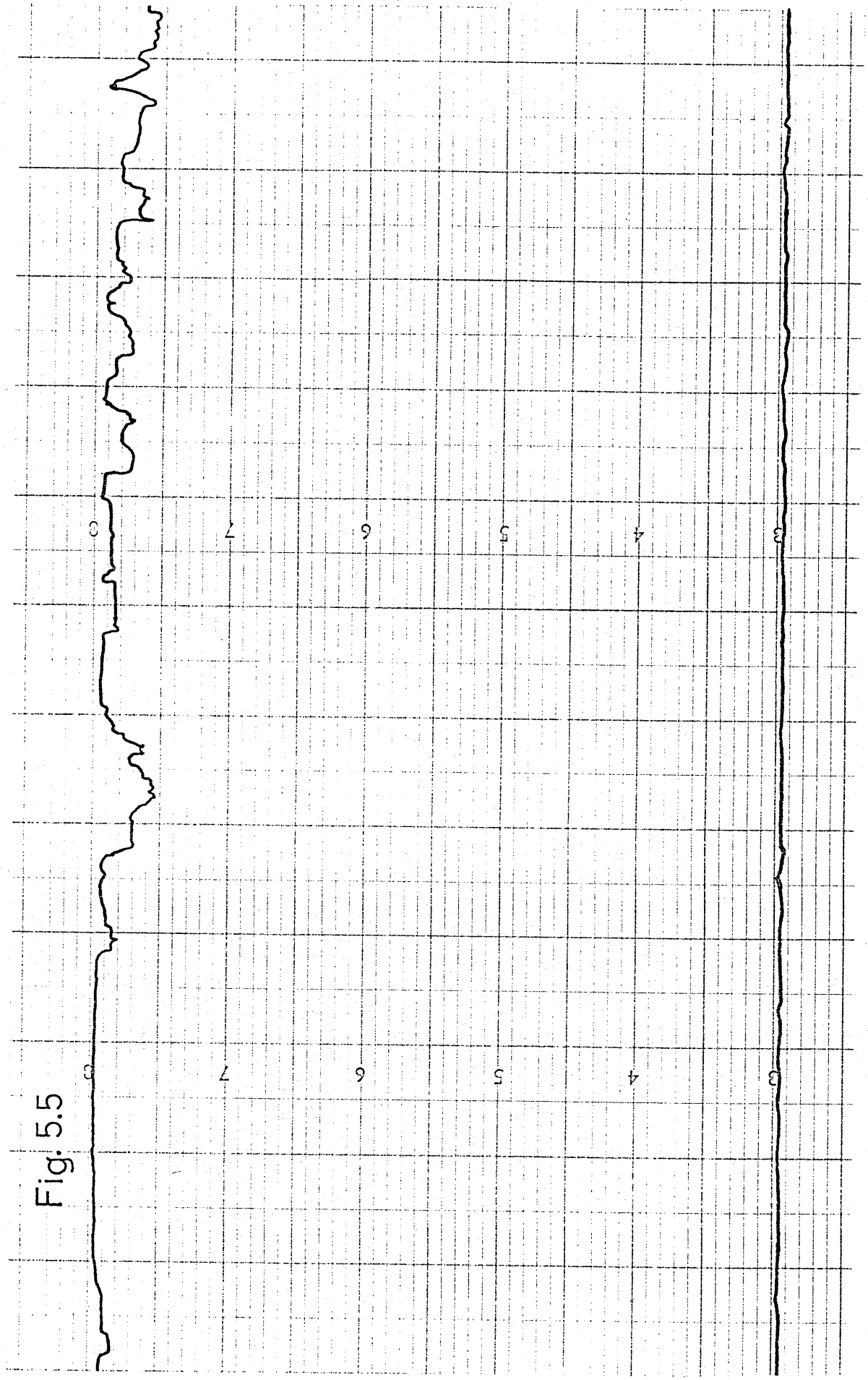


Fig. 5.5

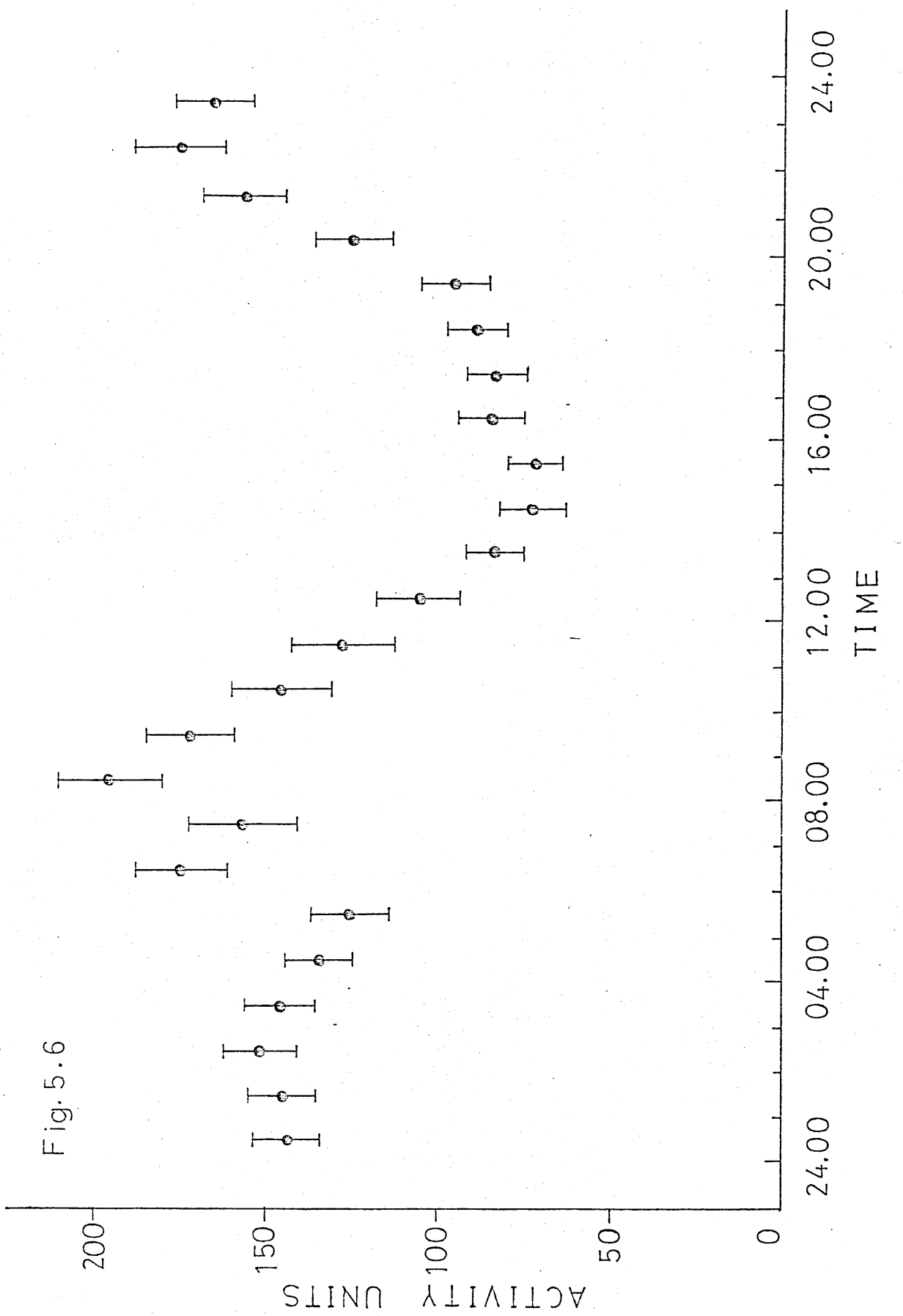
the mean levels of activity between the litters (from 80 activity units to 204 activity units) was attributed largely to the differing numbers of animals per cage recorded. In order to standardize these results to obtain an overall average picture, the following procedure was adopted. The mean of each litter was calculated. The activity units x hours of recording (areas) of the points above the mean and below this mean were calculated. Each of these area figures were then divided by the number of points above and below the mean respectively. The average of these figures for each of the six dark reared litters were taken, and the results of each litter standardized to the same mean figure.

The overall result produced in Fig. 5.6 clearly shows a diurnal rhythmicity reaching a peak at dawn with low levels throughout the day - and in this respect is very similar to the well known behavioural activity cycle of the normally reared rat. The graph indicates that some 67% of the activity occurs during the hours 19.00 to 09.00 hours. A result of the combination and pooling of the data from the six litters would preclude any attempt to, nor was it intended, to establish a precise period for the rhythm - which has previously been shown (Aschoff, 1965) to be slightly extended from 24 h in animals under 'free running' conditions where the activity is not directly driven by environmental lighting conditions.

Figure 5.7 shows for comparison the mean activity levels $\pm$ S.E.M. of 6 litters of normal rats. Each point, calculated in similar fashion to that already described, represents the mean of 10 measurements. The main differences between this record and that from the dark reared animals are illustrated in Fig. 5.8 which plots both sets of results on the same

Legend to Figures 5.6 to 5.8

Overall locomotor activity levels in cages of 50 day old rats over 24 h periods in dark reared unexposed (Fig. 5.6) and normal (Fig. 5.7) animals. Fig. 5.8 represents both groups together. Activity was scored over cumulative 1 h periods and is expressed in arbitrary units as described in the text. Fig. 5.6 depicts the means ± S.E.M. of 23-29 measurements at each point from litters comprising 4-8 animals per cage, and Fig. 5.7 the means ± S.E.M. of 10 measurements at each point from litters comprising 4-8 animals per cage.


ACTIVITY UNITS
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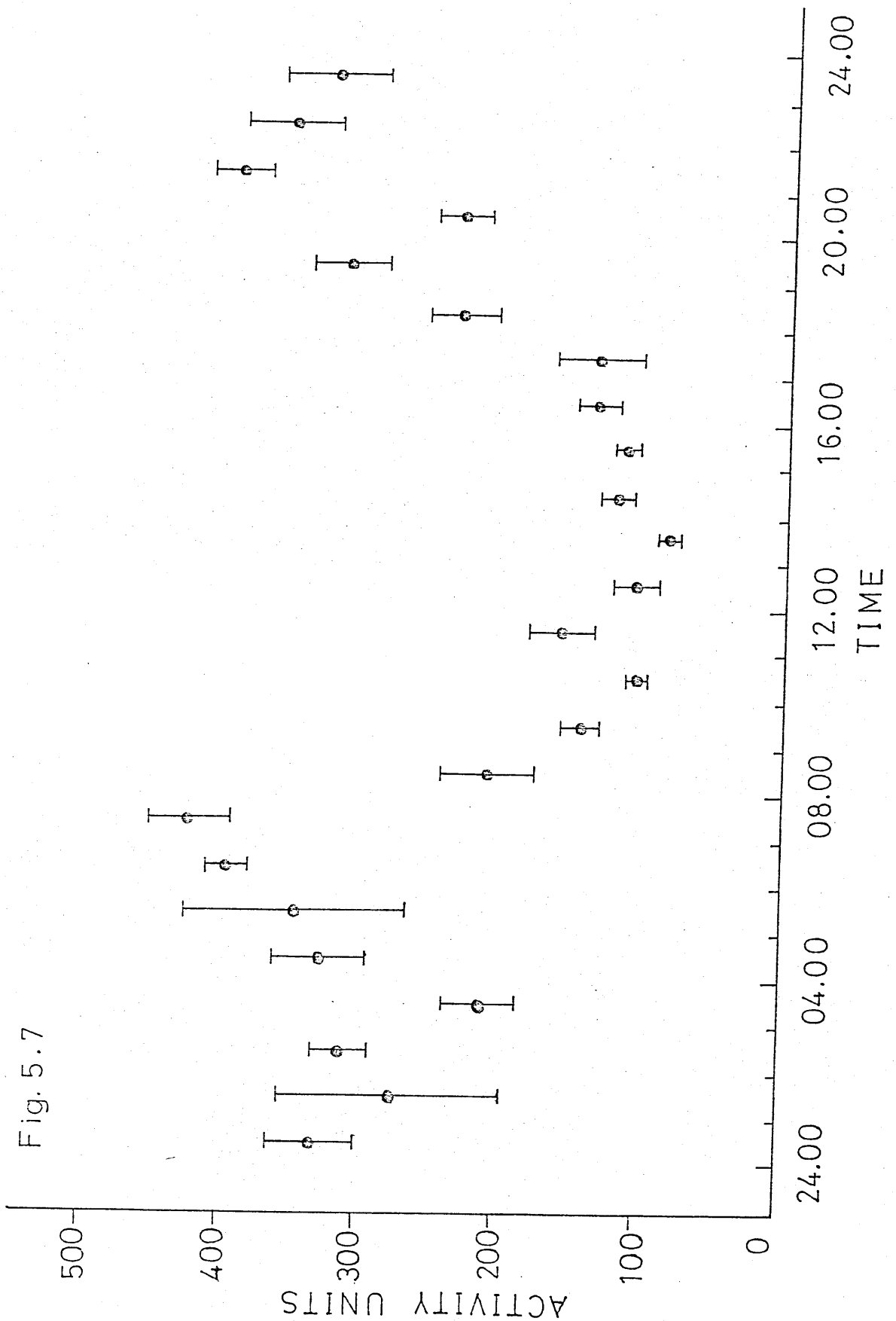
16.00
20.00
24.00
TIME

Fig. 5.6


ACTIVITY UNITS
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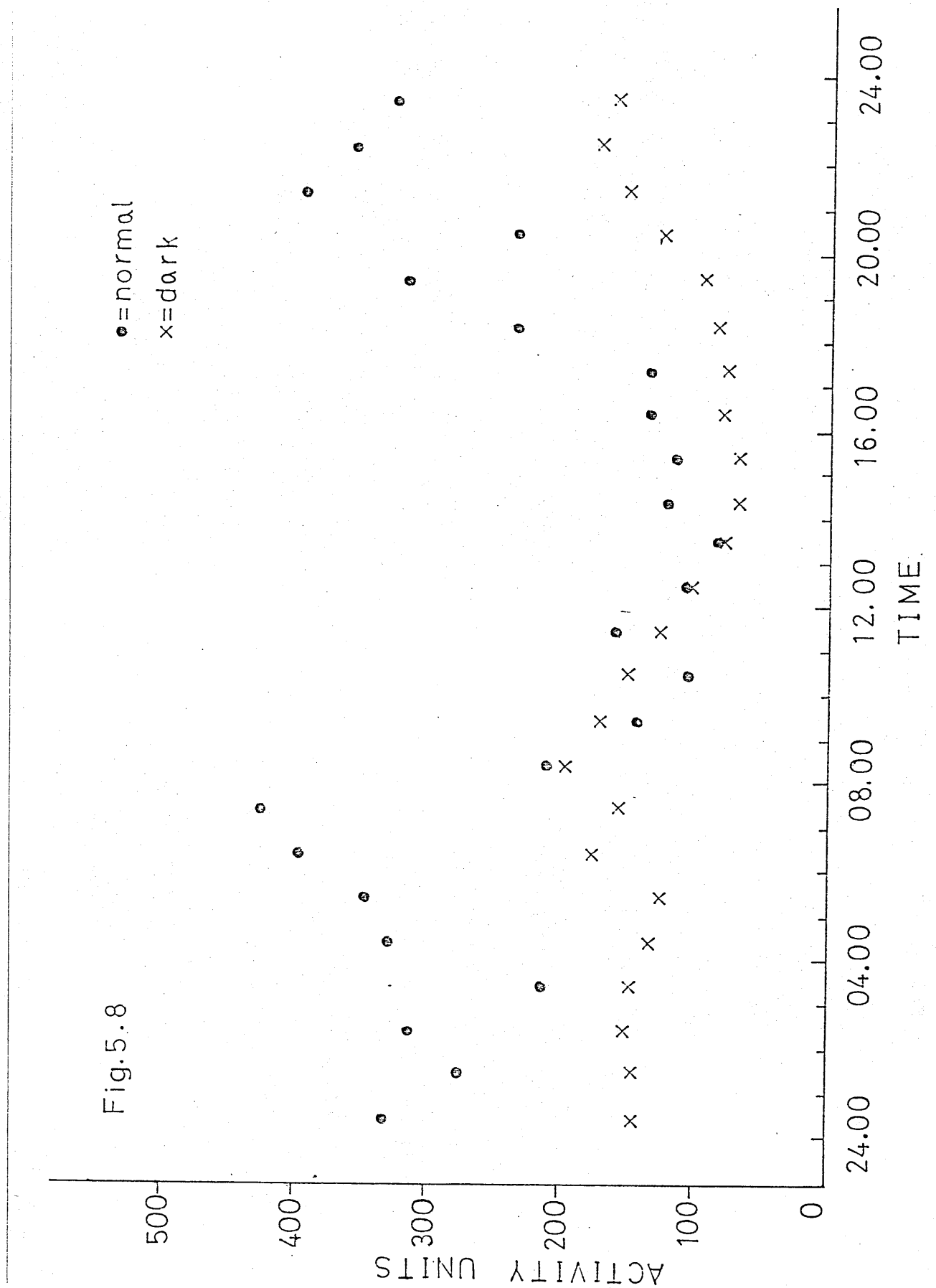
20.00
24.00
TIME

Fig. 5.7


●=normal
x=dark
ACTIVITY UNITS
500
400
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200
100
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24.00
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20.00
24.00
TIME

Fig.5.8

scale (S.E.M's have been omitted here for clarity). The pattern in the normal animal is very similar, with some 80% of the activity occurring during the dark period, as measured by this technique. The immediate difference in the results is that the total level of activity is much greater in the light-driven animals, reaching peaks during the dark phase some 100% higher than the dark reared litters. It is difficult to say with any certainty whether the phase shift of some 1-2 h (15-30^{o}) between the two groups of animals would be significant, and this question would require further detailed investigation to be answered. If the phase shift is not artefactual or spurious, it may be related to a delay in the responsiveness of the dark animals to outside (general animal house) stimulation.

The circadian variation in locomotor activity levels in the dark maintained animals may be compared with the circadian fluctuation in acetylcholinesterase levels in the motor cortex (Fig. 5.3). A comparison of the two sets of data would indicate a fairly close correspondence or correlative relationship, whilst no such obvious correlation is observable with the data from the normal animals.

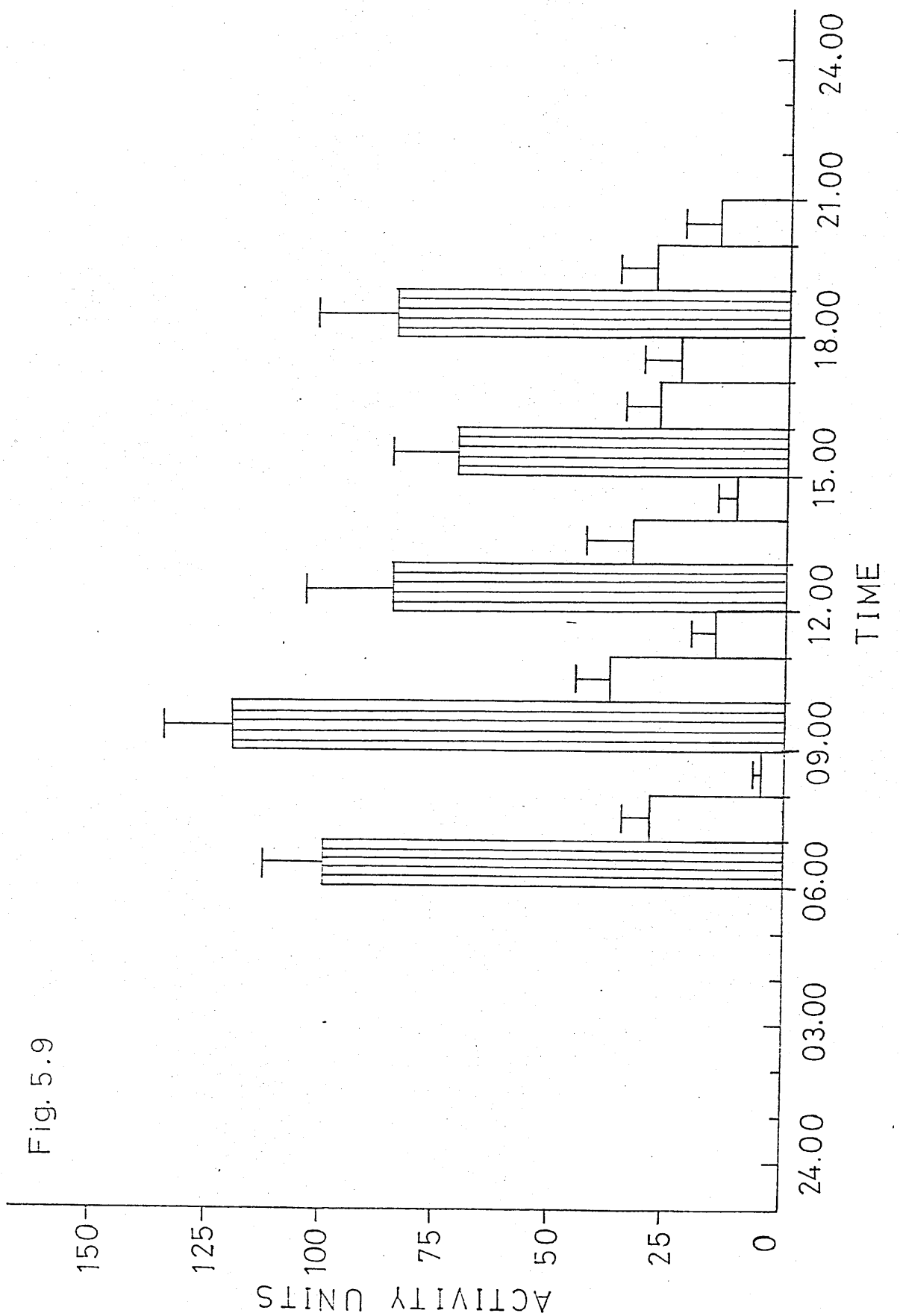
One hypothesis which was advanced earlier in this chapter to account for the time dependent interaction in the elevation of visual cortex acetylcholinesterase levels was that the animals may be less active or behaviourally aroused during light exposure late in the day. This hypothesis was tested by recording the activity of individual animals during light exposure. The results of these experiments are illustrated in Fig. 5.9. The mean number of activity units ± S.E.M. for six measurements at each

hour point are represented hourly during each of the three hour exposure periods commencing at 06.00, 09.00, 12.00, 15.00 and 18.00 h. These experiments utilised different litters from that reported in Fig. 5.3. The results indicate that some 60-75% of the total activity in each exposure period occurs during the initial hour, which is largely accounted for by an extended burst of activity as the animals initially explore their new visual environment and adapt to a one animal per cage situation. The adaptation occurs surprisingly rapidly in view of the sudden and total transformation of visual and social experience. However, this initial spate of activity itself varies cyclically with the time of onset of exposure. This is indicated by comparing the heights of the striped bars in Fig. 5.9. Although the differences are not large in comparison to the variability between the animals, activity during the initial hour after the onset of light is significantly higher during a.m. than p.m. exposure ($t = 2.53$, D.F. = 28, $p < 0.05$).

This result, while not conclusive, provides a clue as to why the elevation in acetylcholinesterase levels which occurs during light exposure is itself time dependent (Fig. 5.1). The animals may simply be less visually attentive when exposure occurs late in the day. To test this hypothesis, a further refinement would be to score the total time the animals spend with their eyes shut and eyes open in each hour period during each of the three hour exposure periods. Unfortunately, a lack of time precluded carrying out this experiment.

Legend to Figure 5.9

The locomotor activity of 50 day old dark reared rats during 3 h light exposure periods at the times shown. Activity was scored over cumulative 1 h periods and is expressed as means ± S.E.M. of arbitrary units from 6 animals at each exposure period. Activity during the initial hour of exposure (striped bars) is higher during a.m. than p.m. exposure ($t = 2.53$, $p < 0.05$). White bars = activity during second and third hours of exposure.


ACTIVITY UNITS
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100
75
50
25
0
24.00
03.00
06.00
09.00
12.00
15.00
18.00
21.00
24.00
TIME

Fig. 5.9

Conclusions

Acetylcholinesterase levels in the visual and motor cortex of normal rats and of rats reared for 50 days in the dark were compared to levels in littermates exposed to three hour periods of visual stimulation during the hours 06.00 to 21.00. An increase in visual but not motor cortex acetylcholinesterase of 21% was observed when exposure occurred during 09.30 to 12.30 and this replicated the observations of Sinha and Rose (1976). No significant increase was detected when exposure occurred later in the day, and it was hypothesised that this was related to a circadian rhythm of visual vigilance. Acetylcholinesterase levels were found to fluctuate with a low amplitude rhythm in the motor, but not in the visual cortex of normal rats and of rats kept in continuous darkness. The locomotor activity of the dark maintained rats was found to vary diurnally with a similar rhythm but lower amplitude to that found in normally reared animals, and this rhythm correlated with the brain motor cortex acetylcholinesterase levels.

CHAPTER SIX

CONTROL OF ACTIVITY DURING LIGHT EXPOSURE

The experiments described in this chapter originally derived from and extend those in Chapter 5, where it was observed that the levels of acetylcholinesterase in the motor cortex of 50 day old dark maintained rats fluctuated in a regular diurnal pattern, and that this was seen to correspond with the amount of spontaneous locomotor activity in the animals. These observations prompted the hypothesis of a correlative relationship between the two measures. One way of testing this would be to observe the effects of involuntary activity on motor cortex enzyme, to determine if the system could be driven. Additionally, the effects of a period of forced activity during the light exposure period on visual cortex enzyme would provide a control and direct test of the hypothesis also advanced in Chapter 5 that the level of behavioural arousal - which may affect visual attention - may be an important variable. It was decided also in these experiments to assay for the more specific cholinergic marker, the synthetic enzyme cholineacetyltransferase, which had also been shown (Sinha and Rose, 1976) to be substantially elevated following a three hour period of light exposure, and also for possible changes in the absolute quantities of the muscarinic cholinergic receptor protein (mAChr) which had recently been found to be elevated some 54% following exposure to light (Rose and Stewart (1978).

Materials and Methods

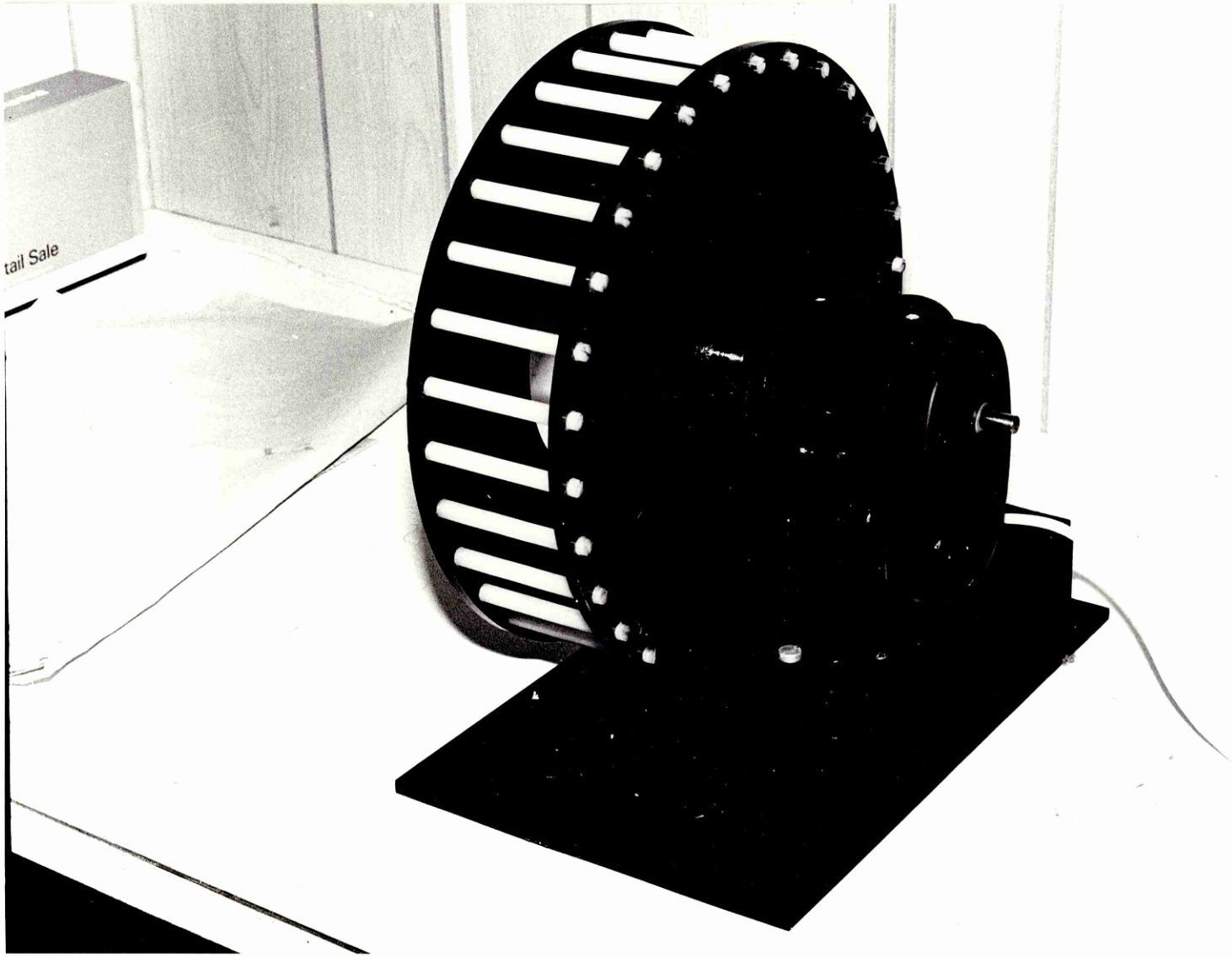
Animals

The animal rearing procedure was as that already described (Chapter 2) with the following differences. Due to a temporary shortage, some of the normally reared animals used in these experiments were purchased from the CFHB Wistar colony of Anglia Animal Suppliers Ltd. (Huntingdon, Cambs.). Normally reared animals of both sexes were used in these experiments. Dark reared animals were used at a range of ages from 30-60 days of age, and the results from each age group are presented separately.

Experimental Procedure

All experiments took place within the same behaviour room previously described (Chapter 5). Some of the dark reared animals, and normal animals of both sexes, were placed in motor driven perspex wheels (diameter = 30 cm) revolving at 2.3 revs/min, corresponding to a rate of movement at the circumference of approximately 4 cm/sec. Plate 4 provides an illustration of one of these wheels. The other dark reared animals and normal animals were placed in a series of identical but undriven wheels on the same bench, which were capable of being self-propelled by the animals. Some experiments were run with the room in total darkness, whilst in others the animals were concurrently exposed to diffuse overhead illumination from tungsten lights (light intensity approximately 200-250 lumens/m^2 at the bench). All experiments were begun between 09.00 and 10.00 hours. Animals were permitted auditory but not visual contact with each other. Following three hours of exposure to their respective conditions, animals were killed by a blow to the back of the

Plate 4. The forced activity apparatus



neck, and visual and motor cortices dissected freehand and placed on ice, exactly as described previously. All dark condition animals were killed in very dim illumination.

Biochemical Procedures

Homogenates of visual and motor cortex were prepared exactly as described previously in Chapter 5. AChE, CAT, ^{3}H QNB specific binding and protein concentration were determined in these homogenates by the methods described in Chapter 4.

Statistical Analysis

A total of 13 dark reared litters and 14 normal litters were used in this series of experiments. The total number of animals in each of the conditions is presented in Tables 6.4, 6.5 and 6.6. As there was some variation in the mean levels of the biochemical parameters measured between the various litters, the results were standardized by the method of constant addition in each case to the grand mean of all the litters for each of the biochemical measures, and subsequent analyses performed on this data. The results from the dark reared animals were analysed with three way (light x activity x age) analyses of variance for each of the biochemical measures for each of the visual cortex and motor cortex results, and also for the ratio of the two (visual/motor cortex). Animals were classed in each of two ways for each of the variables analysed, as follows:-

Light - whether exposed to light or not

Activity - whether exposed to forced activity or not

Age - whether over or under 45 days of age.

In each case animals were classified according to these criteria, irrespective of which other categories were also

applicable. An SPSS Anova programme was used appropriate for a design involving unequal cell frequences, which makes allowance in the variance estimates for the incompletely balanced design. Three-way and higher interaction effects were not computed, and only significant two-way interaction effects are presented in the results. The age classification was chosen as a convenient mid-point separating animals in the range normally used in our experimental design from juvenile animals, whilst insufficient numbers of animals were available in each category for a further accurate break-down of the results into smaller categories. The results from the normally reared animals were analysed in the same cortical areas, using two-way (activity x sex) analyses of variances using the same procedure as described for the dark reared animals. Significance of differences between these categories were subsequently calculated using the Student's t test (2 tailed probability values).

Results and Discussion

Tables 6.1, 6.2 and 6.3 summarise the results of the analyses for each of the biochemical measures. Light exposure has significant effects in the visual cortex in all three measures, but not in the motor cortex, and in the AChE and ^{3}H QNB binding data this also achieves significance in the visual/motor ratio. There are also very significant effects related to the age of the animals on all three measures, but since these do not interact with either of the two experimental treatments (exposure or activity) the effects of these treatments are not differential between the two age groups. Male animals differed significantly from female animals in both cortical areas, but only in CAT activity, and there were also significant activity x sex interaction effects

in this biochemical measure. The effects of the forced activity are significant on motor cortex AChE and ^{3}H QNB specific binding for the dark reared animals, but not in CAT activity, and these are also significant in the visual/motor cortex ratio. There were no significant effects of the activity in normally reared animals.

Tables 6.1 to 6.3 summarize the analyses of these differences, and Tables 6.4 to 6.6 represent the results of the t test comparisons between the individual groups. Due to the design employed in these experiments, where some animals were light exposed under conditions of simultaneous activity whilst others were not, and all animals had free locomotion restricted, these results are not necessarily directly comparable to those obtained with our normal experimental design. For example, no allowance has been made for possible stressful artefacts introduced under these conditions. As indexed by amount of defecation, which in the rat is commonly taken as a crude index of the amount of trauma (Paré, 1964) and which increases when animals are placed in these wheels (N. Wood, unpublished observations) possible confounding effects of stress cannot be dismissed.

Nevertheless, the results generally reflect and extend those previously found (see Table 6.7). The light induced elevation in visual cortex acetylcholinesterase is again replicated, as is a larger increase in ^{3}H QNB specific binding to mAChr originally reported by Rose and Stewart (1978). The light induced elevation in visual cortex CAT activity was only 6% which is, surprisingly, significant (Table 6.5) with the low variability found in this measure - although this

TABLE 6.1 Analysis of variance summary table of acetylcholinesterase results

(a) Dark reared animals

REGION	MAIN EFFECTS			LIGHT EFFECTS			ACTIVITY EFFECTS			AGE EFFECTS		
	D.F.	F	p	D.F.	F	p	D.F.	F	p	D.F.	F	p
Visual cortex	3	28.78	0.001	1	4.76	0.033	1	0.66	n.s.	1	5.68	0.001
Motor cortex	3	14.22	0.001	1	0.41	n.s.	1	5.61	0.021	1	36.31	0.001
Visual/motor cortex	3	6.74	0.001	1	3.65	0.06	1	7.31	0.009	1	0.86	n.s.

(b) Normal animals

REGION	MAIN EFFECTS			ACTIVITY EFFECTS			SEX EFFECTS		
	D.F.	F	p	D.F.	F	p	D.F.	F	p
Visual cortex	2	0.65	n.s.	1	0.002	n.s.	1	1.30	n.s.
Motor cortex	2	1.32	n.s.	1	0.24	n.s.	1	2.34	n.s.
Visual/motor cortex	2	0.02	n.s.	1	0.03	n.s.	1	0.005	n.s.

The Table indicates the results of 3-way (2 light conditions x 2 activity conditions x 2 age groups) analyses for dark reared animals and 2-way (2 activity conditions x 2 sexes) analyses for normally reared animals on acetylcholinesterase data from each brain region. There were no significant interactions. SPSS Anova (unequal cell frequencies).

TABLE 6.2 Analysis of variance summary table of cholineacetyltransferase results

(a) Dark reared animals

REGION	MAIN EFFECTS			LIGHT EFFECTS			ACTIVITY EFFECTS			AGE EFFECTS		
	D.F.	F	p	D.F.	F	p	D.F.	F	p	D.F.	F	p
Visual cortex	3	9.88	0.001	1	5.59	0.022	1	1.24	n.s.	1	21.48	0.001
Motor cortex	3	13.91	0.001	1	0.008	n.s.	1	1.37	n.s.	1	40.57	0.001
Visual/motor cortex	3	1.41	n.s.	1	2.39	n.s.	1	0.02	n.s.	1	2.04	n.s.

(b) Normal animals

REGION	MAIN EFFECTS			ACTIVITY EFFECTS			SEX EFFECTS			ACTIVITY x SEX INTERACTIVE EFFECTS		
	D.F.	F	p	D.F.	F	p	D.F.	F	p	D.F.	F	p
Visual cortex	2	27.43	0.001	1	1.68	n.s.	1	52.07	0.001	1	2.90	0.092
Motor cortex	2	16.66	0.001	1	1.18	n.s.	1	32.68	0.001	1	3.96	0.05
Visual/motor cortex	2	2.26	n.s.	1	2.03	n.s.	1	2.26	n.s.	1	5.37	0.024

The Table indicates the results of 3-way (2 light conditions x 2 activity conditions x 2 age groups) analyses for dark reared animals and 2-way (2 activity conditions x 2 sexes) analyses for normally reared animals on cholineacetyl-transferase data. Only significant interactions are indicated. SPSS Anova (unequal cell frequencies).

TABLE 6.3 Analysis of variance summary table of ^{3}H QNB binding results

(a) Dark reared animals

REGION	MAIN EFFECTS D.F.	F	p	LIGHT EFFECTS D.F.	F	p	ACTIVITY EFFECTS D.F.	F	p	AGE EFFECTS D.F.	F	p
Visual cortex	3	10.71	0.001	1	4.46	0.041	1	0.20	n.s.	1	27.46	0.001
Motor cortex	3	13.23	0.001	1	2.51	n.s.	1	5.7	0.023	1	31.47	0.001
Visual/motor cortex	3	6.124	0.002	1	12.3	0.002	1	6.07	0.019	1	0.001	n.s.

(b) Normal animals

REGION	MAIN EFFECTS D.F.	F	p	ACTIVITY EFFECTS D.F.	F	p	SEX EFFECTS D.F.	F	p
Visual cortex	2	0.093	n.s.	1	0.18	n.s.	1	0.0	n.s.
Motor cortex	2	1.74	n.s.	1	1.43	n.s.	1	2.42	n.s.
Visual/motor cortex	2	2.14	n.s.	1	2.62	n.s.	1	2.13	n.s.

The Table indicates the results of 3-way (2 light conditions x 2 activity conditions x 2 age groups) analyses for dark reared animals and 2-way (2 activity conditions x 2 sexes) analyses for normally reared animals on ^{3}H QNB binding data. There were no significant interactions. SPSS Anova (unequal cell frequencies).

TABLE 6.4 Table of acetylcholinesterase t value comparisons. Ratio = visual/motor cortex

COMPARISON	REGION	N1/N2	t VALUE	D.F.	p VALUE	N1(n)	N2(n)
N1 = light exposed	Visual	1.17	3.68	50	0.001	28	24
N2 = dark control	Motor	1.02	0.37	49	n.s.	28	23
	Ratio	1.20	3.26	50	0.002	28	24
N1 = dark reared and active	Visual	1.09	1.58	50	n.s.	13	39
	Motor	0.92	1.62	49	n.s.	13	38
N2 = dark reared not active	Ratio	1.21	3.09	50	0.003	13	39
N1 = normal and active	Visual	1.0	0.05	54	n.s.	15	41
N2 = normal, not active	Motor	0.98	0.55	53	n.s.	15	40
	Ratio	1.03	0.53	54	n.s.	15	41
N1 = 45-60 days old	Visual	1.31	8.50	50	0.0001	28	24
N2 = 30-45 days old (All dark reared)	Motor	1.22	5.58	49	0.0001	27	24
	Ratio	1.05	0.72	50	n.s.	28	24
N1 = females	Visual	1.03	1.08	54	n.s.	24	32
N2 = males (All normals)	Motor	1.05	1.56	53	n.s.	24	31
	Ratio	1.03	0.51	54	n.s.	24	32

TABLE 6.5 Table of cholinacetyltransferase t value comparisons
Ratio = visual/motor cortex

Comparison	Region	N1/ N2	t value	D.F.	p value	N1(n)	n2(n)
N1 = light exposed	Visual	1.06	2.28	45	0.027	28	19
	Motor	1.01	0.38	45	n.s.	28	19
N2 = dark control	Ratio	1.04	1.50	45	n.s.	28	19
N1 = dark reared, and active	Visual	0.99	0.20	45	n.s.	11	36
	Motor	0.98	0.58	45	n.s.	11	36
N2 = dark reared, not active	Ratio	1.01	0.45	45	n.s.	11	36
N1 = normal and active	Visual	0.95	1.13	45	n.s.	13	34
	Motor	1.02	0.63	46	n.s.	13	35
N2 = normal, not active	Ratio	0.96	0.56	46	n.s.	13	35
N1 = 45-60 days old	Visual	1.10	4.81	45	0.0001	23	24
	Motor	1.14	6.42	45	0.0001	23	24
N2 = 30-45 days old	Ratio	0.97	1.26	45	n.s.	23	24
(All dark reared)							
N1 = females	Visual	1.22	7.09	45	0.0001	20	27
N2 = males	Motor	1.15	5.55	46	0.0001	20	28
(All normals)	Ratio	1.10	1.67	46	n.s.	20	28

TABLE 6.6 Table of ^{3}H QNB binding t value comparisons
Ratio = visual/motor cortex

Comparison	Region	N1/N2	t value	D.F.	p value	N1(n)	N2(n)
N1 = light exposed	Visual	1.31	2.13	35	0.05	16	21
	Motor	0.93	0.98	35	n.s.	17	20
N2 = dark control	Ratio	1.21	1.94	36	n.s.	17	21
N1 = dark reared and active	Visual	1.08	0.93	35	n.s.	9	28
	Motor	0.83	2.11	35	0.042	9	28
N2 = dark reared, not active	Ratio	1.35	2.96	36	0.005	9	29
N1 = normal and active	Visual	0.99	0.14	42	n.s.	13	31
	Motor	0.95	1.09	39	n.s.	13	28
N2 = normal not active	Ratio	1.37	2.32	44	0.025	13	33
N1 = 45-60 days old	Visual	1.33	4.86	35	0.0001	21	16
	Motor	1.34	5.06	35	0.0001	21	16
N2 = 30-45 days old	Ratio	1.11	1.03	36	n.s.	21	17
(All dark reared)							
N1 = females	Visual	1.0	0.0	42	n.s.	20	24
N2 = males	Motor	0.93	1.52	39	n.s.	18	23
(All normal)	Ratio	1.19	1.27	44	n.s.	20	26

increase recedes into insignificance on the unstandardized raw data. This increase is not consistent with the 30% increase in visual cortex and 22% increase in motor cortex enzyme obtained after a similar exposure period by Sinha and Rose (1976) and this may be related to the present differences in experimental design. There were no changes in CAT activity in respect of the other experimental manipulation, forced exercise, in either the dark reared or normally reared animals under these conditions. A significant decrease in ^{3}H QNB binding in the motor cortex and ratio results of the dark reared animals following this experimental treatment was however discovered, and which in the visual/motor cortex ratio results is also reflected in a reduction in acetylcholinesterase. No effects were found in the motor cortex on either measure following this treatment in normally reared animals, although a similar alteration in the visual/motor cortex ratio results is reflected at least in the ^{3}H QNB binding data. This latter result, from a t test comparison, should however be regarded with some caution as the analysis of variance (Table 6.3b) had failed to reveal this as a significant effect.

Figs. 6.1 (AChE), 6.2 (CAT) and 6.3 (QNB) summarize graphically the size of these changes related to light exposure and forced activity in the dark reared and normally reared animals. Each bar represents the mean ± S.E.M. of the results of the visual/motor cortex calculations, enabling a direct comparison to be made between the three measures.

The data (combined from all experimental treatments) was also broken down dichotomously by age of animals into categories of 30-45 days old or 45-60 days old (dark reared

TABLE 6.7 Summary of statistically significant differences from Tables 6.4 - 6.6

Region	Comparison	AChE	CAT	^{3}H QNB
Visual	Light exposed/darks	1.17	1.06	1.31
Cortex	Over 45 days/under 45 days	1.31	1.10	1.33
	Females/males		1.22	
Motor	Active/inactive dark reared			0.83
Cortex	Over 45 days/ under 45 days	1.22	1.14	1.34
	Females/males		1.15	
Visual/Motor	Light exposed/darks	1.20		
Cortex	Active/inactive dark reared	1.21		1.35
	Active/inactive normals			1.37

animals only) and by sex (normal animals only). A significant reduction in all three cholinergic markers is present in animals in the younger age group. Figs. 6.4 (AChE results in µm of substrate hydrolysed/min/mg protein, 6.5 (CAT results in DPM/mg protein/h) and 6.6 (^{3}H QNB results in pmoles bound/mg protein) present graphically a further breakdown of this data in animals grouped at 5 day intervals (categories absent reflect missing data). This developmental profile of an age dependent increase in cholinergic activity in our dark reared animals is in agreement with results for AChE obtained in normally reared animals by Elkes and Todrick (1955) and Adlard and Dobbing (1970) and for AChE and CAT in normally reared and dark reared animals by Bigl and Biesold (1978).

Only in CAT activity, however, were there any significant differences related to the sex of the (all normal) animals, with female animals showing a 15-22% increase over the male animals. Figs. 6.7 (AChE in µm substrate hydrolysed/min/mg protein), 6.8 (CAT in DPM/mg protein/h) and 6.9 (^{3}H QNB in pmoles bound/mg protein) summarize the data for the visual and motor cortex broken down by sex.

The differences obtained in this series of experiments which are related to the experimental treatments are suggestive, but also raise a number of questions. The results would indicate that our dark reared animals can differ significantly from normally reared rats, not only in respect of the well documented changes in the visual system, but in other aspects of brain-behaviour interactions. The dark reared animals, as discussed in Chapter 5, are around 50% spontaneously less active than similar cages of normal animals,

Legend to Figures 6 .1 to 6.3

The effects of 3 h periods of light exposure and forced activity on 30-60 day old dark reared and normally reared rats for levels of acetylcholinesterase (Fig. 6.1) cholineacetyltransferase (Fig. 6.2) and ^{3}H QNB specific binding (Fig. 6.3). Each bar represents the mean ± S.E.M. of 9-41 animals and is expressed as the ratio of visual cortex levels/ motor cortex levels. Numbered bars represent as follows:

1. Dark reared and not exposed to light, in stationary wheels
2. Dark reared and exposed to light in stationary wheels
3. Dark reared and exposed to light in motor driven wheels
4. Normally reared and placed in stationary wheels
5. Normally reared and placed in motor driven wheels

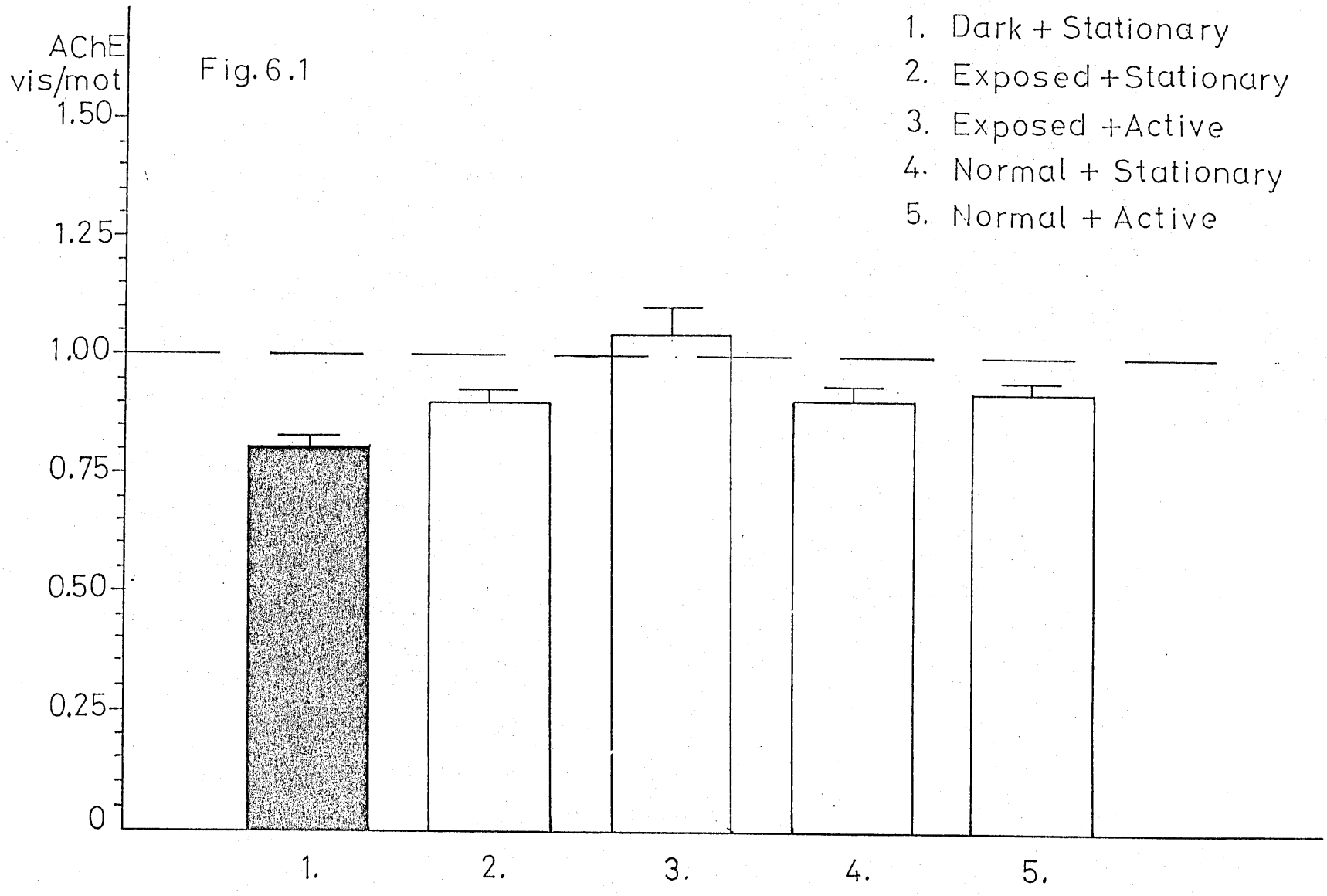

AChE
vis/mot
1.50
1.25
1.00
0.75
0.50
0.25
0
1.
2.
3.
4.
5.
1. Dark + Stationary
2. Exposed + Stationary
3. Exposed + Active
4. Normal + Stationary
5. Normal + Active

Fig. 6.1

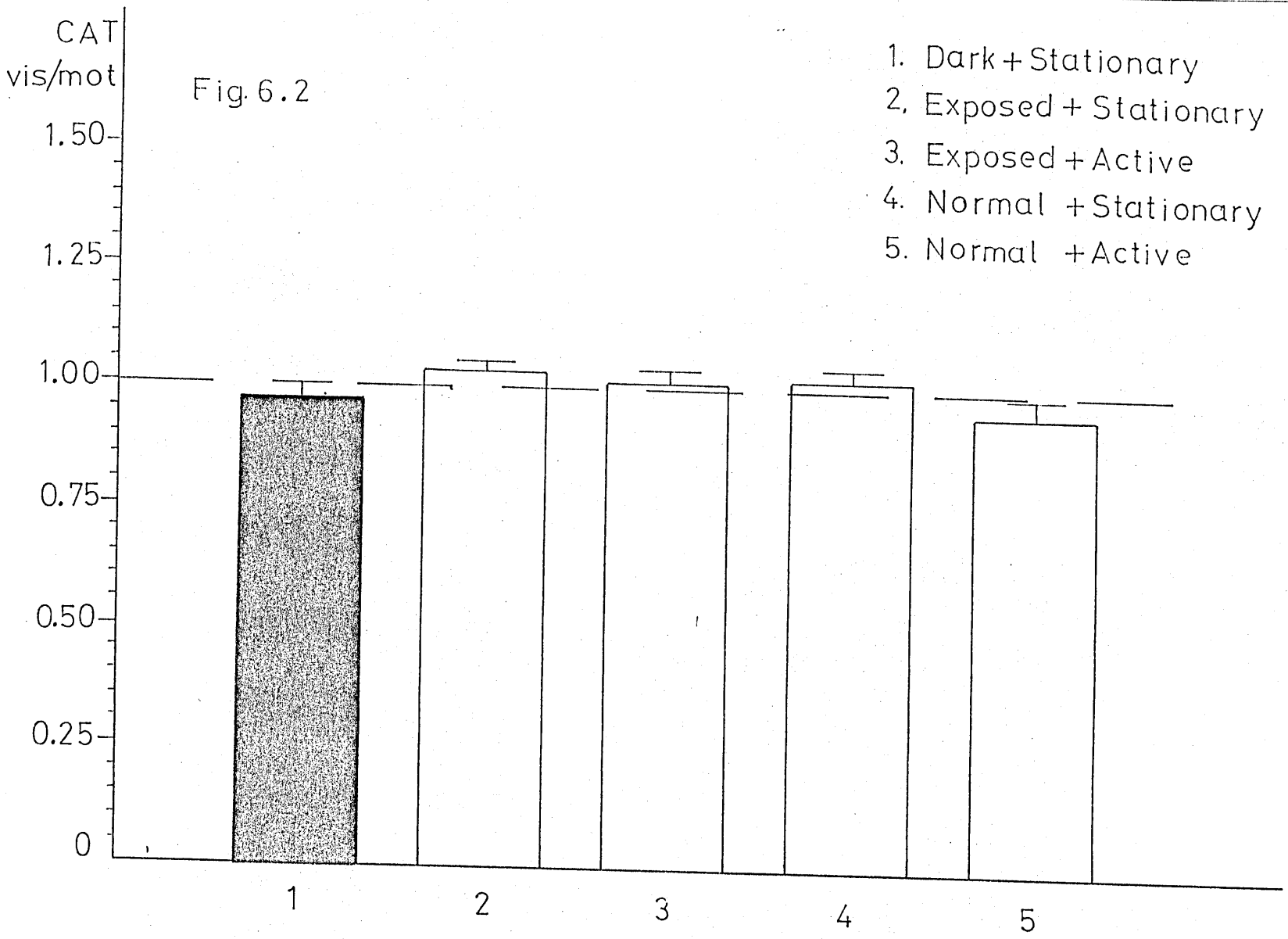

CAT
vis/mot
1.50
1.25
1.00
0.75
0.50
0.25
0
1
2
3
4
5
1. Dark + Stationary
2. Exposed + Stationary
3. Exposed + Active
4. Normal + Stationary
5. Normal + Active

Fig. 6.2

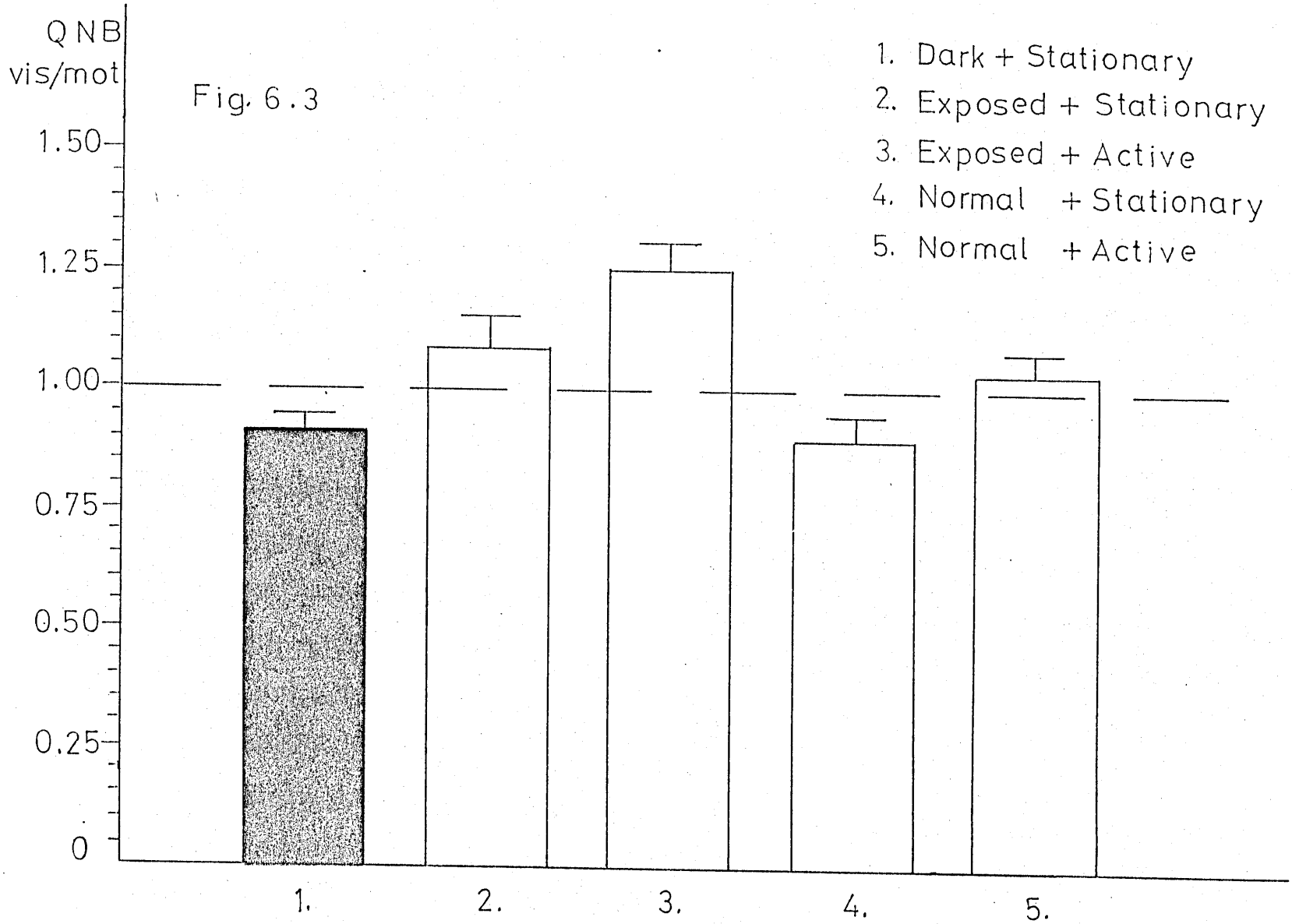

QNB
vis/mot
1.50
1.25
1.00
0.75
0.50
0.25
0
1.
2.
3.
4.
5.
1. Dark + Stationary
2. Exposed + Stationary
3. Exposed + Active
4. Normal + Stationary
5. Normal + Active

Fig. 6.3

Legend to Figures 6.4 to 6.6

The effects of age of dark reared animals grouped at 5 day intervals between the ages of 30-60 days on measured acetylcholinesterase (Fig. 6.4), choline-acetyltransferase (Fig. 6.5) and ^{3}H QNB specific binding (Fig. 6.6) in visual (white bars) and motor (striped bars) cortex. Categories absent reflect missing data. AChE activity is expressed as µmoles of substrate hydrolysed/min/mg protein, CAT activity as DPM/mg protein/h x 10^{-4} and ^{3}H QNB binding as pmoles bound/mg protein,. Each bar represents the mean ± S.E.M. of 6-21 animals.

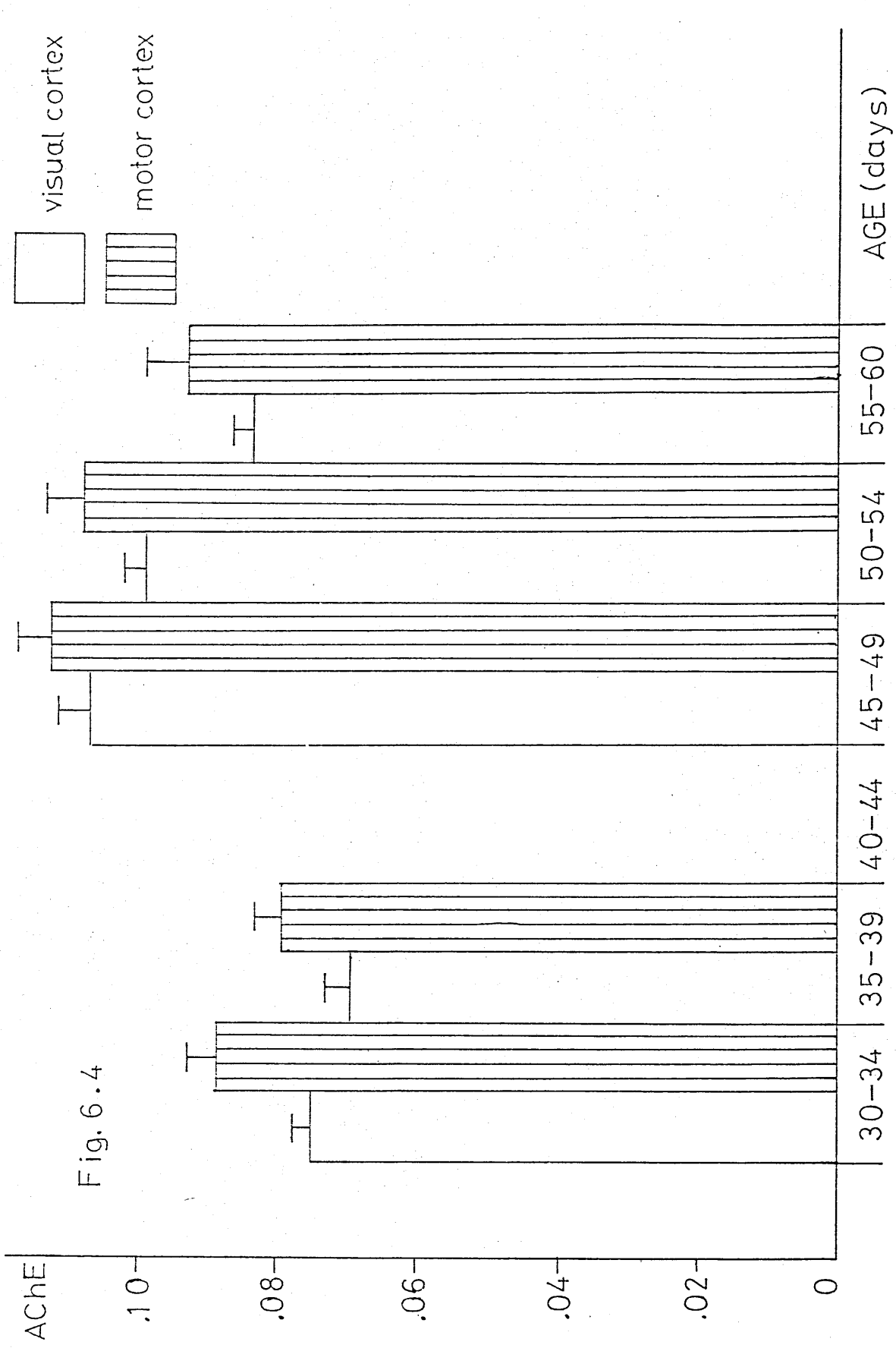

visual cortex
motor cortex
ACh E
.10
.08
.06
.04
.02
0
30–34
35–39
40–44
45–49
50–54
55–60
AGE (days)

Fig. 6.4

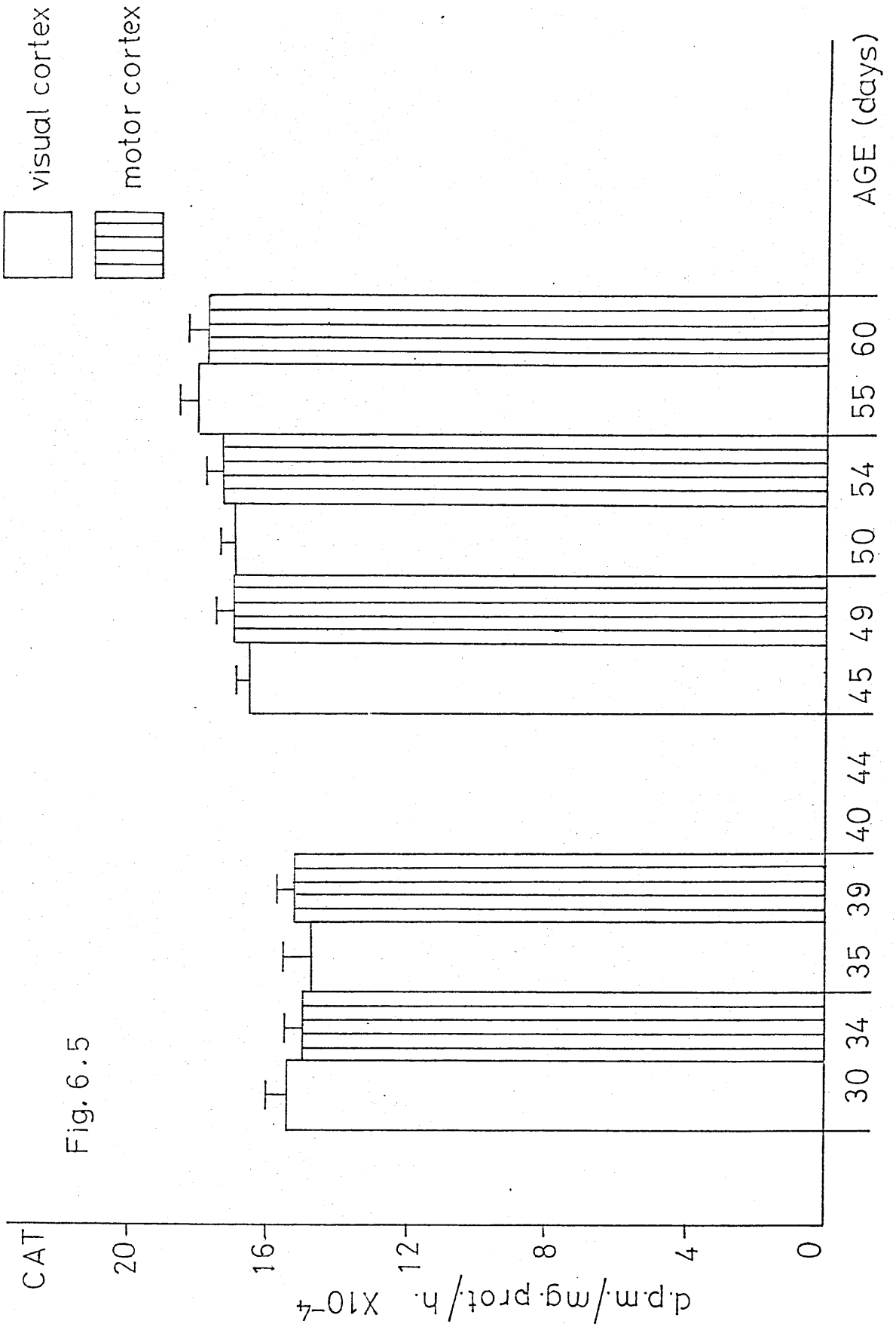

visual cortex
motor cortex
CAT
d.p.m./mg.prot./h. X10⁻⁴
0
4
8
12
16
20
30 34 35 39 40 44 45 49 50 54 55 60
AGE (days)

Fig. 6.5

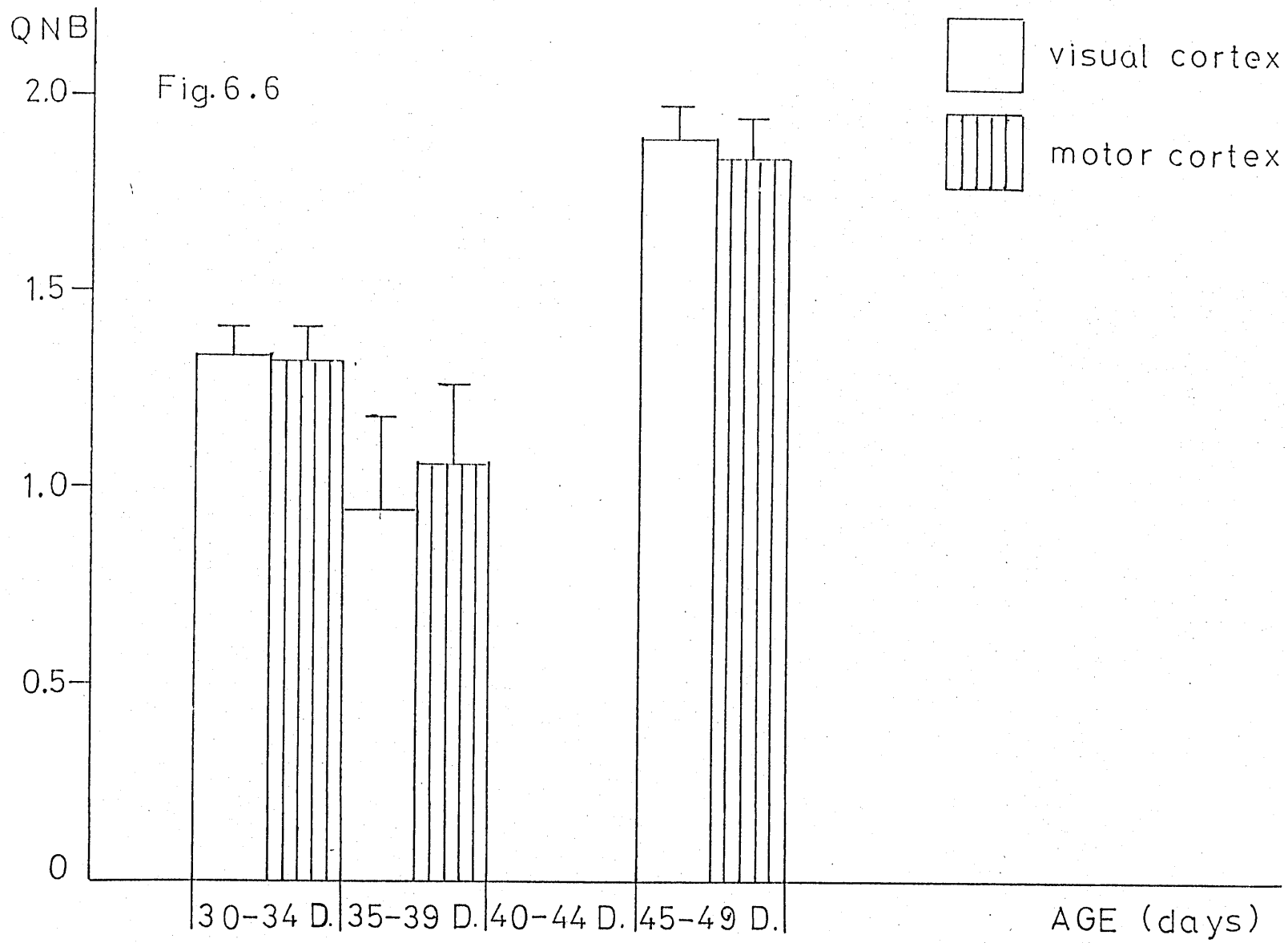

QNB
2.0
1.5
1.0
0.5
0
visual cortex
motor cortex
30-34 D.
35-39 D.
40-44 D.
45-49 D.
AGE (days)

Fig. 6.6

Legend to Figures 6.7 to 6.9

Acetylcholinesterase (Fig. 6.7), cholineacetyl-transferase (Fig. 6.8) and ^{3}H QNB specific binding (Fig. 6.9) in visual and motor cortex of female (striped bars) and male (white bars) normally reared rats. AChE activity is expressed as μmoles of substrate hydrolysed/min/mg protein, CAT activity as DPM/mg protein/h x 10^{-4} and ^{3}H QNB binding as pmoles bound/mg protein. Each bar represents the mean ± S.E.M. of 18-32 measurements. CAT female/male ratio in visual cortex = 1.22 (t = 7.09, $p < 0.0001$) and in motor cortex = 1.15 (t = 5.55, $p < 0.0001$).

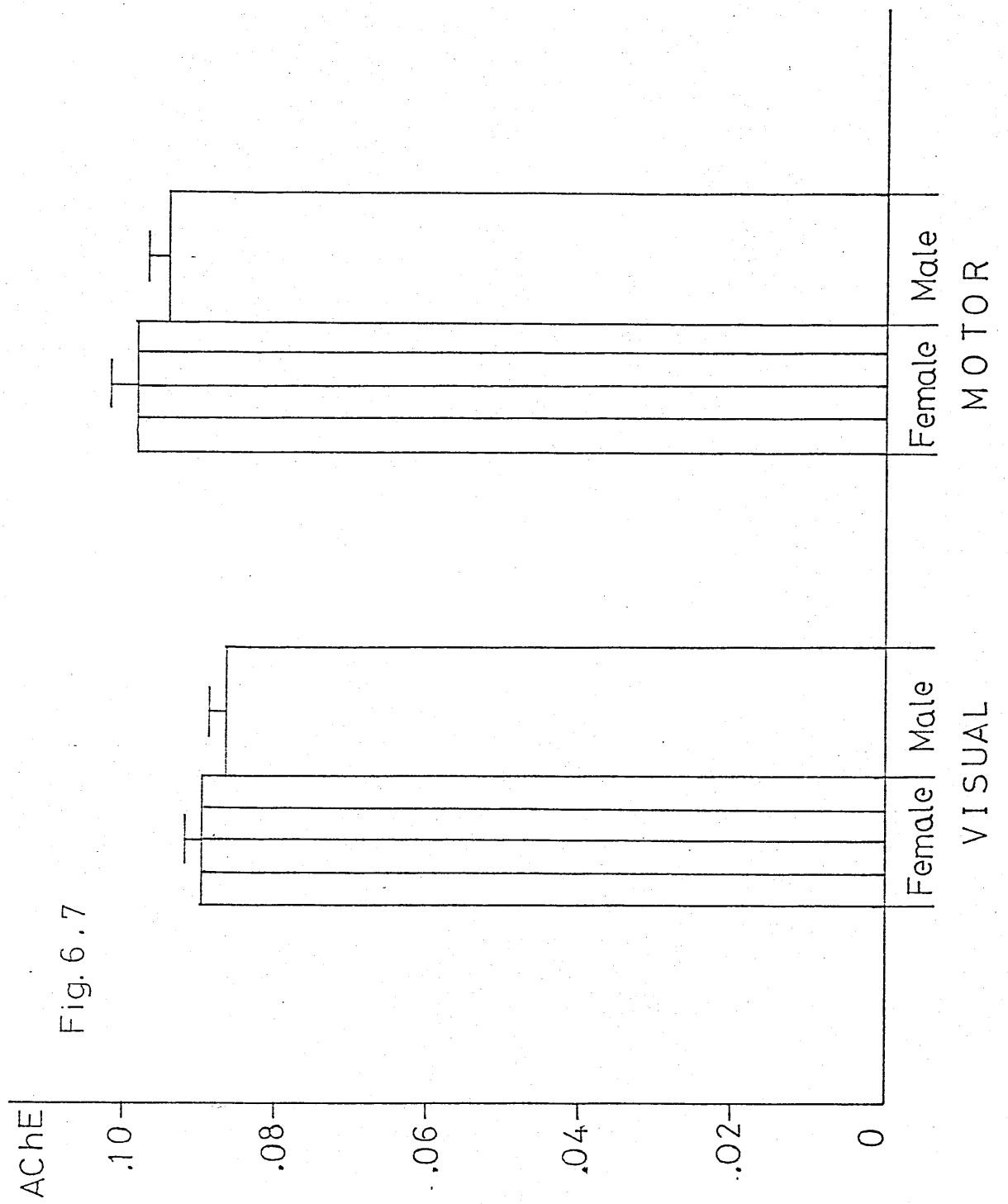


Fig. 6.7

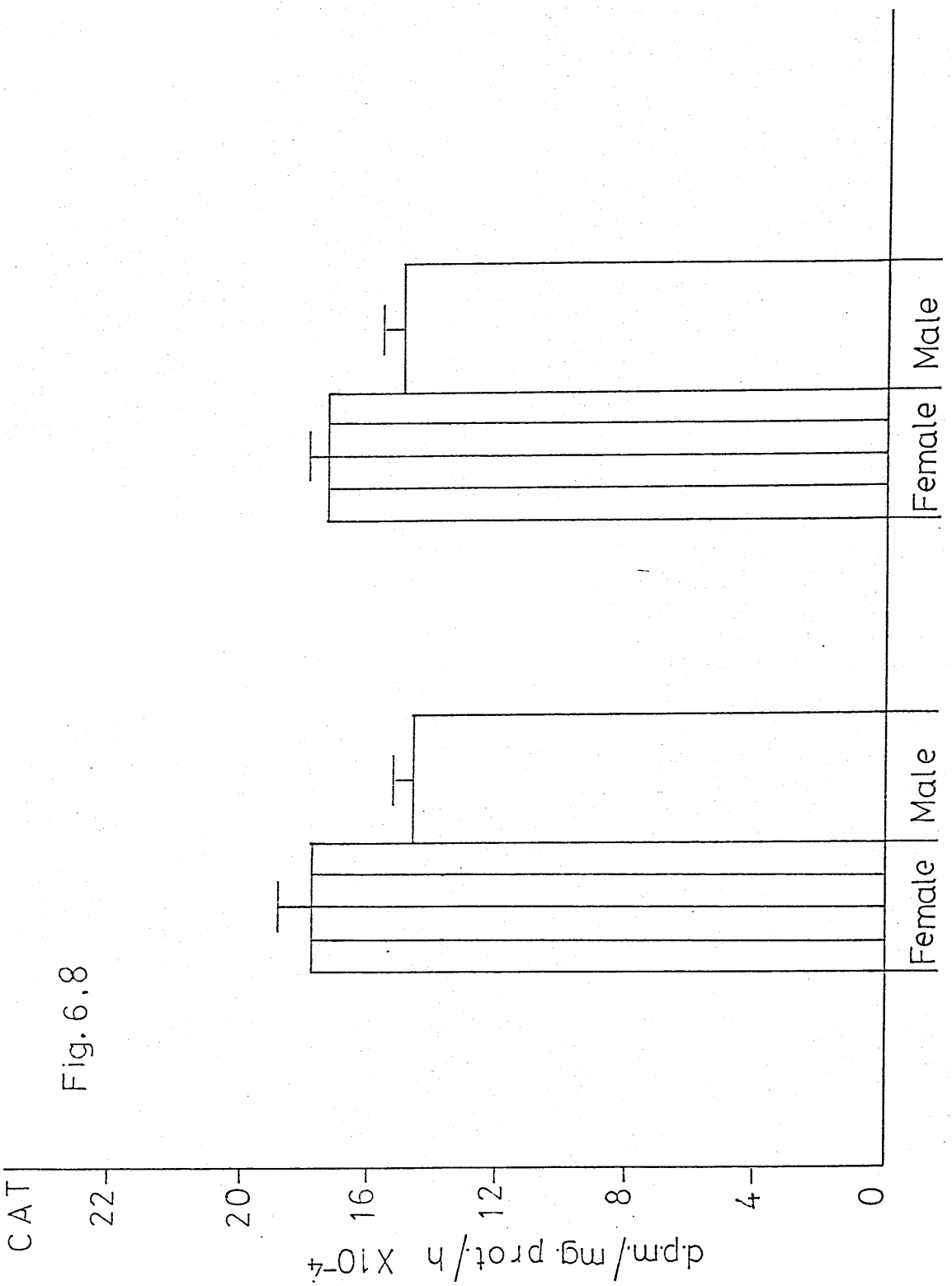

CAT
d.p.m./mg.prot./h X10⁻⁴
0
4
8
12
16
20
22
Female
Male
VISUAL
Female
Male
MOTOR

Fig. 6.8

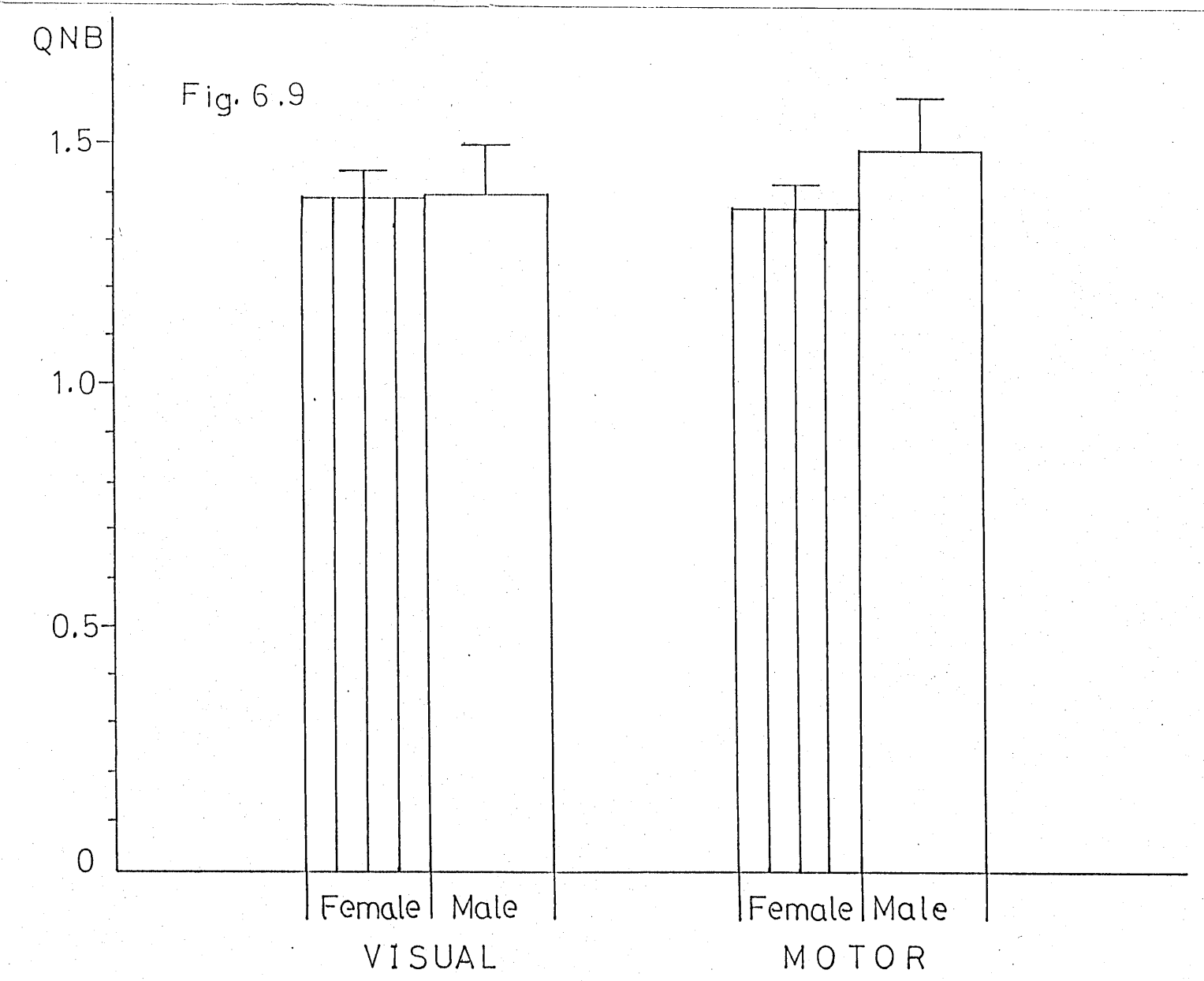


Fig. 6.9

and it appears that the experimentally induced activity has a correspondingly differential effect. As was also discussed in Chapter 5, the light deprived animals display a diurnal rhythmicity in AChE levels which tends to parallel the pattern of spontaneous locomotor behaviour, whereas normally reared animals show no simple correspondence. As a tentative explanation for this difference, it is possible that 'driving' the motor cortex is effectively acting, even in a relatively short period, to specify otherwise developmentally delayed unspecific pathways leading to reductions in AChE and mAChr as a process of synaptic stabilisation (Changeux and Danchin, 1976) with appropriate input to these pathways.

The largest differences, both in the visual cortex following visual experience and in the motor cortex following artificially induced activity, are observed in ^{3}H QNB specific binding , and these are generally reflected in similar but smaller (and with less variability in the data) changes in AChE levels. This is consistent with current knowledge of the topographical linking of esterase and receptor on the post-synaptic membrane. Unlike the enzyme activity measurements, those of mAChr involve a more direct assay of the number of receptor molecules, or binding sites, present. No such large differences were observed in CAT levels, however, following either experimental treatment. It is entirely feasible that, for example, changes in ACh turnover could be reflected by alterations in degradative rate not reflected in changes in synthetic rate, during the periods of functional stimulation. The regulation rate and mechanisms of the CAT enzyme remains unfortunately less than completely understood. Rat brain CAT has been shown to consist of three

molecular forms (Malthe-Sorensen and Fonnum, 1972), all of which have been isolated from perikarya and axons as well as from the synaptosomes (Fonnum and Malthe-Sorensen, 1973). Additionally, alterations in the amount of brain CAT or AChE found at any point of time following functional stimulation might be due to the changes in underlying dynamic processes of synthesis, degradation or activation, and thus may represent only the end point of different functional events. Some evidence as to the nature of the biochemical control processes involved has been provided (Rose, 1978a). To distinguish between the alternative possibilities that these changes in ^{3}H QNB binding activity and enzyme levels, following light exposure, result either from an increase in the synthesis of new molecules or from the activation or unmasking of pre-existing precursor molecules, experiments involving simultaneous light exposure in animals in which protein synthesis has been attenuated by anisomycin (40 mg/kg injected i.p.) indicate that the elevation in ^{3}H QNB binding capacity is not present (S.P.R. Rose, unpublished). While suggestive, these preliminary experiments are not conclusive, as the anisomycin blocked only around 60-70% of the total cerebral protein synthesis, whilst behaviourally, preliminary experiments indicate (S.P.R.Rose and N. Wood, unpublished) that the total spontaneous locomotor activity of anisomycin injected animals may be reduced by about one third, and hence the interpretation is not straightforward.

To turn to the behavioural questions, the results discussed in Chapter 5 would not predict a depression in AChE contingent upon the forced exercise, for when spontaneous locomotor behaviour was high, so were acetylcholinesterase levels. One

possible explanation for this is that spontaneous activity occurs within normally defined limits, whereas our 3 h period of forced activity, although comparatively mild, represents an abnormal period of a prolonged behaviour pattern amounting to a novel experiential situation, to which the animal must learn to adapt. The behavioural response to the experience is a stereotyped one - typically the animal will run a few steps, be carried round with the wheel for a few seconds, and then run a few more steps, usually remaining at the base of the wheel. This would seem to be the best available strategy to minimise the work expenditure. (Animals will sometimes cling on to the rungs and be carried bodily round with the wheel, but this presumably vestibular-stimulating exercise is not usually prolonged). Speculatively, it may be hypothesised that responses in the synaptic pathways involved may become less flexible and more specificed with time. Indeed, due to the considerable redundancy in the neuronal network, it may reduce the total number of active pathways involved. This specificiation could account for the reductions in AChE and ^{3}H QNB binding observed, whereas the fact that CAT remains constant would point to no overall changes in the enzymically regulated synthesis of the transmitter.

It should be noted that these results are consistent with previous reports of neurochemical alterations associated with a period of forced exercise, although the evidence is contradictory. Using normally reared animals, Tiplady (1972) observed a 15-20% decrease in the rate of incorporation of a precursor (^{3}H lysine) into rat cerebral cortex protein following periods of activity in a motor driven wheel, and that this was not due to an impairment of the protein

synthetic capacity of the cell (Tiplady et al., 1973). Jakoubek and Gutmann (1968) observed a decrease in the rate of incorporation of labelled amino acid into spinal motoneurones after 90 min forced swimming. In contrast, Altman (1967) found an increase of incorporation of precursor into protein in all brain regions associated with a period of forced exercise in an activity wheel, though this was determined autoradiographically. It may also be noted that Rose (1967) observed a decrease in incorporation of ^{3}H lysine into motor cortex proteins of some 16% following a 3 h period of light exposure, and an increase of 18% in motor cortex protein incorporation following a 3 h period of forced activity in a rotating wheel. Some of these contradictory results may be resolved if the behavioural variables, and in particular the measures employed to control for aspects of differential stress were better defined. It has been shown for example (Jakoubek et al., 1970) that a decrease in the incorporation rate into protein can occur solely as a result of anticipation stress. Although these results cannot be compared directly with those reported in this chapter, the behavioural problems are similar.

Conclusions

Rats were reared for a range of ages from 30 to 60 days in darkness, and subsequently exposed to three hour periods of light alone or with simultaneous mild but continuous activity in a motor driven wheel. The results indicate that the light-induced elevations in visual cortex AChE levels and ^{3}H QNB binding are still present in animals exposed under these conditions, although only a very small increase in visual cortex CAT activity was observed under these conditions. There were no changes in CAT in either visual or motor cortex associated with the other

functional stimulus, the forced activity, but there were significant effects in motor cortex AChE and ^{3}H QNB specific binding. Normally reared animals displayed a different pattern of changes associated with the forced activity. All three cholinergic markers were significantly reduced in animals in the younger half of the age group studied in comparison to older animals, confirming results from other laboratories, and there was a signficant difference between normally reared male and female animals of comparable ages, but only in CAT levels.

CHAPTER SEVEN

CONTROL OF VISUAL EXPERIENCE DURING LIGHT EXPOSURE

Although the regionally specific light induced elevations of enzyme activity in the light exposed rats have so far been implicitly associated with functions of the altered stimulus situation related directly to visual experience, it has not been clear which observed biochemical changes are associated with which aspects of the total environmental stimulus. Thus, unlike the situation in the young chick (Rose, 1978b) where control experiments have indicated that enhanced incorporation of ^{14}C uracil into RNA in a forebrain area can be directly attributed to the learning of an imprinting stimulus (an orange flashing light), no biochemical changes yet studied following the first exposure of dark reared rats to the light have been thus far defined. It has not been ascertained for example, which, if any, of the changes are related to the onset of perceptual function in the visual system or to associated cortical events involving the acquisition and intersensory integration (cross-modal linkage) of events in the novel sensory modality. By analogy with the chick imprinting experiments, brain processes underlying such changes should be neurochemically distinguishable from any arising from straightforward visual sensory stimulation.

The experiments described in this chapter were undertaken, using a single biochemical measure - acetylcholinesterase activity - in an approach to distinguishing effects related to general visual stimulation from those related to information processing in the cortex. The nature of the visual experience during light exposure was controlled by the use of light diffusing contact occluders, which effectively prevent patterned light from striking the retinae.

Materials and Methods

Contact occluders

The contact occluders were constructed by a modification of the method of Clamp (personal communication). Lenses were scleral contacting of the type described by Bonnet (1973), fitting under the eyelid and in close contact with the entire corneal surface. Plates 5 and 6 are macrophotographs illustrating these lenses in place. Plate 5 illustrates a rat with a transparent lens fitted, showing the visibility of the iris, and Plate 6 illustrates the opaque occluders in place. Some surface irregularities are present, and some loss of optical definition may have occurred, but for these experiments an appropriate control procedure was adopted (see below).

Lenses were shaped to the rat's eye by the following procedure. Male rats in the weight range 160-250 g at 10 g intervals were selected. The eyes were excised by manual protrusion from the socket and optic nerve excision with curved iris scissors. The freshly excised eyes were immersed in a mixture of New Kromopan alginate impression material (Wright Dental Group, Dundee) made in a proportion of 0.3 g/ml water. After solidification, the eyes were removed and a positive cast made by introducing into the mould a mixture of dental stone (G. Nissel and Co., Hemel Hempstead) made in a proportion of 3.0 g/ml water. After hardening, the positive casts were examined for flaws. Approximately 2.5 cm^2 pieces of 0.25 mm cellulose acetate were cut from transparent, light diffusing and opaque (black) sheets, and warmed on a heated plate to approximately 150^{o} C. The now pliable plastic was placed over the positive casts and pressed down with a 0.9 cm diameter hollow tube. After cooling, the edges were trimmed and rounded to shape.

Plate 5. Transparent lens in situ

Plate 6. Opaque occluders in situ

A range of lenses were thereby constructed which were matched to animals within 5 g body weight. These were placed on to the eyes of the lightly ether anaesthetised animal after instilling 2 drops of 0.1% amethocaine as a local anaesthetic and wetting agent on to the corneal surface.

Animals

The animal rearing procedure was exactly as that previously described in Chapter 2. All experiments subsequently took place within the same behaviour room as previously described (Chapter 5) with the following experimental design.

Exeperimental Procedure

11 separate litters of dark reared animals with their normally reared littermates were used in this series of experiments. The normal animals, after brief ether administration as a control procedure, were placed in the individual transparent plastic cages. The dark reared animals were randomly assigned to each of 5 conditions as follows, after removal from the rearing box and a 60-90 s (according to response) exposure to diethyl ether in darkness:

1. Condition L. Animals were placed in individual transparent plastic cages.

2. Condition CL. Clear transparent plastic occluders were binocularly fitted, after which animals were placed in the exposure cages.

3. Condition TL. As (2) except that light diffusing occluders were fitted.

4. Condition DL. As (2) except that opaque occluders were fitted.

5. Condition D. Animals were placed in individual cages inside a dark box.

The first four of these groups of animals, together with the normal animals (N) were exposed to overhead illumination from a tungsten light (luminous flux approximately = 200-260 lumens/m^2 at the bench). Condition TL was exposed in addition to a further overhead light which had been determined to be sufficient to compensate for the reduced light intensity striking the eye, as measured through an unfitted diffusing lens with a CdS light meter. All animals were exposed adjacently on the same bench, and were permitted auditory but not visual contact with each other. Commencement of exposure was between 09.00 and 10.00 h in all cases, to avoid the complications due to diurnal rhythmicity discussed in Chapter 5. Following three hours of exposure to their respective conditions, animals were killed by a blow to the back of the neck, and visual and motor cortices rapidly dissected freehand as previously described (Chapter 2) and placed on ice.

Biochemical Procedure

Homogenates containing approximately 15-20 mg tissue/ml were prepared as described previously (Chapter 5) and acetylcholinesterase activity was determined on freshly homogenized tissue by the method of Ellman *et al.* (1961), exactly as described in Chapter 4. Duplicate 100 µl aliquots of the homogenate were taken for protein determination by the method of Lowry *et al.* (1951), using a BSA standard as previously described (Chapter 4).

Statistical Procedure

All AChE results were expressed as µmoles of substrate hydrolysed/min/mg protein, and calculated as means ± S.E.M. of the animals in each condition. As an alternative to standardization of results, an analysis of variance procedure was adopted in which litter variables were separately analysed and

corrected for in the analysis. As the number of animals per litter varied from 5-9, in each experiment an unequal number of animals were present in each condition, and an appropriate method to allow for this unbalanced design was selected (SPSS Anova programme, unequal cell frequencies). Separate analyses of variance were run for the visual cortex, motor cortex, and visual/motor ratio results. Only primary and 2-way interaction effects were computed. Animals were categorised according to litter number (I-II) type of light condition (conditions L and CL being regarded as equivalent and D and DL as equivalent), and whether wearing lenses or not (irrespective of light condition). Three-way (4 light conditions x 2 lens conditions x II litters) analyses were therefore computed. This anova procedure was initially adopted to safeguard against the possibility that chance statistically significant differences become more likely in comparisons between six sets of data from two brain regions. Significance of differences between conditions were subsequently determined using the students t test (2 tailed probability values).

Results and Discussion

Fig. 7.1 shows the results of preliminary control experiments performed to assess the effects of the ether administration procedure on measured AChE levels. Each column represents the mean ± S.E.M. of the results from 4-6 animals. Littermate animals were chosen, half of which were exposed to diethyl ether for 90 s and killed either 5 min or 3 h afterwards, subsequent procedures being performed as for the experimental animals. Control littermate animals were treated similarly with no ether exposure. The results indicate no significant differences in measured AChE levels between the two groups of

animals in either brain region after either 5 min or 3 h. However, a trend to a depression in cortical AChE levels is detectable after 5 min (visual cortex, $t = 1.545$). Neglecting the unlikely possibility of an interaction between one experimental treatment rather than another, following the administration of ether during the experimental procedure, it was assumed that as all animals, whether wearing lenses or not, had similar ether exposure, the differences reported are due to the experimental manipulations alone.

The results of the analysis of variance procedure are summarized in Table 7.1. As there were no significant interaction effects, these are excluded from the Table. The effects of the lighting variable are significant on AChE levels in the visual cortex ($F = 5.17$, $p < 0.02$) but not in the motor cortex ($F = 1.1$). Litter variability contributes a significant proportion of the total variance in both the visual ($F = 3.87$, $p < 0.01$) and motor cortex ($F = 4.55$, $p = 0.005$) data, but not in the ratio of the two. This latter result indicates that, although the mean levels of enzyme vary significantly between litters in each of the brain regions, the proportion in the two brain areas is relatively constant (with higher AChE activity being found in the motor cortex); nor were there any significant interactions found between litters and the light exposure variable. This latter result indicates that, although individual animals can - indeed do - vary in their response to light exposure, there are no consistent litter-orientated effects other than in the mean levels of brain enzyme. On the assumption that total genetic pool variance between litters is larger than that within litters, even in our closely inbred colony, this result would point to little effect of any genetic differences

Legend to Figure 7.1

The effect of a control 90 s diethyl ether administration on acetylcholinesterase (μmoles of substrate hydrolysed/min/mg protein) levels in visual and motor cortex of 50 day old normal rats, determined 5 min and 3 h after administration. Each bar represents the mean ± S.E.M. of 4-6 animals exposed to ether (striped bars) or non-exposed littermates (white bars).

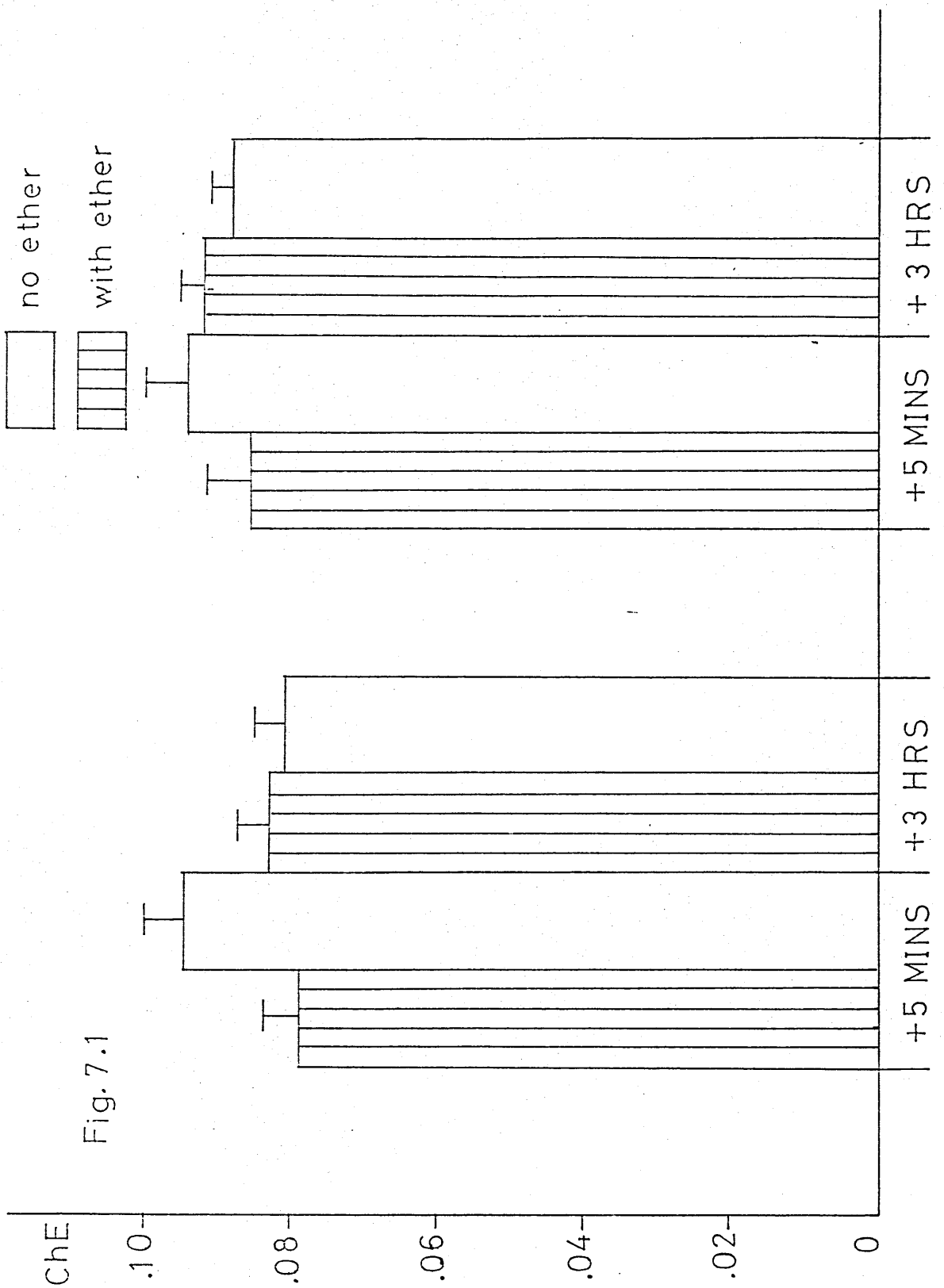

no ether
with ether
AChE
.10
.08
.06
.04
.02
0
+5 MINS
+3 HRS
VISUAL CORTEX
+5 MINS
+3 HRS
MOTOR CORTEX

Fig. 7.1

between litters influencing the neurochemical responses to initial visual experience. The outcome of this analysis also provides further statistical affirmation of the procedure of standardization of results based around litters which has previously been employed in this study, and in our laboratory generally.

Table 7.1 also indicates that the presence of the lenses per se (over all light exposure conditions) provides no systematic effect on the data, and this was regarded as an essential control procedure. It is not ruled out that the close-fitting occluders may induce some level of stress; if this is the case,however, it has not acted to affect the present results. In view of this, for purposes of further analysis, condition CL can be regarded as equivalent to condition C,and condition DL to condition D.

Table 7.2 provides a summary tabulation of the enzyme activity measurements ± S.E.M. in each of the conditions, together with the occluder types associated, and Fig. 7.2 provides a graphical indication of these figures. The overall elevation in visual cortex enzyme in the animals exposed to the patterned light over the dark controls is 15% ($t = 2.88$, D.F. = 33, $p < 0.01$) and the elevation in the diffuse light exposed animals was some 17% ($t = 3.45$, D.F. = 29, $p < 0.01$). The equivalent comparisons in the motor cortex are not statistically significant in either case, although a trend to an increase was observed (5% increased in the patterned light exposed animals and 6% in the diffuse light exposed). This trend to an increase in motor cortex enzyme, although small and insignificant statistically, was also noted in the experiments described in Chapter 5, and was also reported by

TABLE 7.1 Analysis of variance summary table

Region	Main effects			Light effects			Litter effects			Lens effects		
	D.F.	F.	p.	D.F.	F.	p.	D.F.	F.	p.	D.F.	F.	p.
Visual cortex	14	4.11	0.005	3	5.17	0.012	10	3.87	0.009	1	0.09	n.s.
Motor cortex	14	3.63	0.009	3	1.10	n.s.	10	4.55	0.005	1	0.88	n.s.
Visual/ motor cortex	14	0.73	n.s.	3	1.46	n.s.	10	0.58	n.s.	1	0.38	n.s.

The table indicates the results of three-way (4 light conditions x 2 lens conditions x 11 litters) analyses in each brain region for acetylcholinesterase data. There were no significant interactions. SPSS Anova (unequal cell frequencies).

TABLE 7.2

Acetylcholinesterase activity in visual and motor cortex of normal, patterned, and diffuse light exposed and unexposed littermate rats.

Condition	Occluder type	Visual cortex activity	Motor cortex activity
Light exposed L	None	0.0753 ± 0.0033	0.0830 ± 0.0034
Light exposed CL	Clear	0.0733 ± 0.0046	0.0760 ± 0.0044
Light exposed TL	Diffusing	0.0757 ± 0.0027	0.0801 ± 0.0035
Dark control D	None	0.0641± 0.0020	0.0764± 0.0035
Dark control DL	Opaque	0.0654 ± 0.0029	0.0754 ± 0.0039
Normal N	None	0.0686 ± 0.0024	0.0726 ± 0.0041

Enzyme activity is expressed as μmoles substrate hydrolysed/min/mg protein. Results are means ± S.E.M. of 11 experiments involving 11 separate litters each of 5-9 animals.

Legend to Figure 7.2

Acetylcholinesterase levels (μmoles of substrate hydrolysed/min/mg protein) in visual and motor cortex of 50 day old dark reared rats exposed to 3 h periods of diffuse and patterned light, in dark reared and unexposed, and in normally reared littermates. Each bar represents the mean ± S.E.M. of 8-15 animals.

Numbered bars represent as follows:-

1. Light exposed with no lenses] = patterned light exposed
2. Light exposed wearing transparent lenses]
3. Light exposed wearing light diffusing lenses = diffuse light exposed
4. Dark controls placed in opaque boxes]
5. Dark controls, light exposed, wearing opaque lenses] = unexposed
6. Normally reared light exposed littermates

Patterned light exposed/unexposed = 1.15 ($t = 2.88$, $p < 0.01$) in visual cortex.

Diffuse light exposed/unexposed = 1.17 ($t = 3.45$, $p < 0.01$) in visual correx.

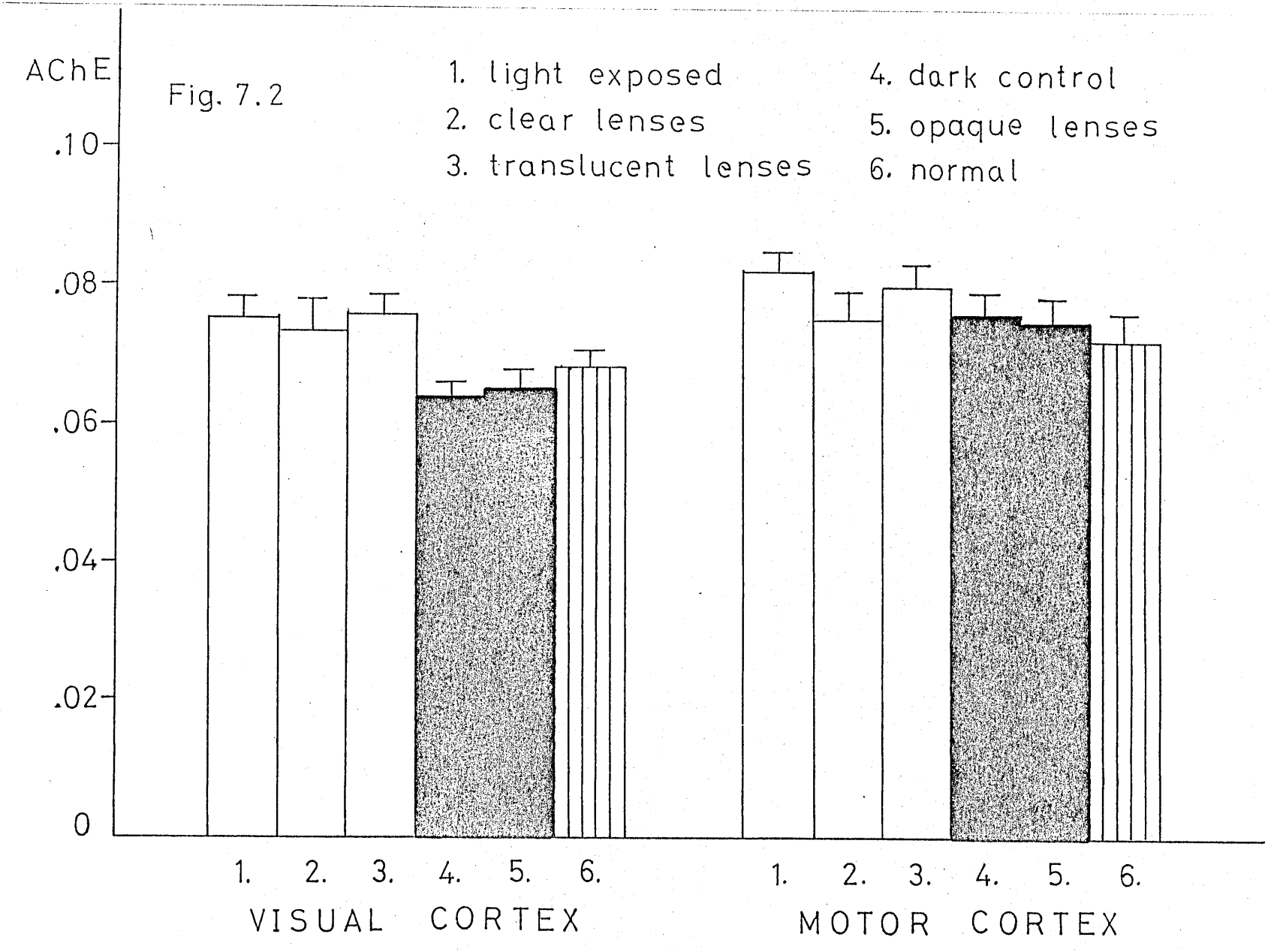

AChE
.10
.08
.06
.04
.02
0
1. light exposed
2. clear lenses
3. translucent lenses
4. dark control
5. opaque lenses
6. normal
1. 2. 3. 4. 5. 6.
VISUAL CORTEX
1. 2. 3. 4. 5. 6.
MOTOR CORTEX

Fig. 7.2

Sinha and Rose (1976). The possible significance of this trend will be discussed later. The AChE activity in either brain region in normally reared animals is once again not significantly different from that of their dark reared and unexposed littermates.

In the terms of the question which was posed at the beginning of this chapter, and which these experiments were designed to elucidate, what aspects of the new stimulus situation for the light exposed animals may be salient in the alteration of AChE activity in the visual cortex? Condition DL, in which animals wearing opaque lenses were exposed to light and were also unlike the normal dark controls previously used in that they were not isolated in the physical enclosure of a box, provides one control. As enzyme activity is not elevated in this group, this indicates the effectiveness of the occluders. and provides more direct evidence than has previously been obtained that the enzyme changes are related to the visual experience solely and not, for example, to any non-specific aspects of the stimulus situation such as possible altered auditory input, temperature change, air currents or olfactory stimulation.

What aspects of the new visual experience may then be important? It has been shown (Miller and Cooper, 1974) that the application of light diffusing contact occluders results in a failure of the normal hooded rat, using a training procedure, to discriminate between simple visual stimuli which were matched for total brightness. Although our animals have not been tested for visual ability wearing these occluders, and despite possible differences in the nature of the present occluders, with the known lesser visual acuity of the albino

rat (Munn, 1950) and after a period of dark rearing, it is at least a safe inference that the processes of visual perceptual learning must be much reduced in the presence of the light diffusing occluders compared at least to the potential opportunities in the two normal light exposed conditions. Nevertheless, the elevation in acetylcholinesterase is still present. The results from these experiments then provide no direct support for a hypothesis that the changes observed in visual cortex AChE are directly related to processes of perceptual learning. However, with this essentially negative result, nor can the opposite conclusion be assumed. Whilst visual stimulation as an explanation is not ruled out, it is not necessarily nor even probably indicated to the exclusion of an alternative explanation. Table 7.3 provides some possible behavioural interpretations of the changes observed in visual cortex AChE in each of the 5 experimental conditions for the dark reared animals. This has been compiled from the known visual abilities of normal, dark reared and occluded rats. These interpretations can only be regarded as tentative, as differences in length of visual deprivation and strain differences may make extrapolation to our colony less than fully appropriate. Rose (1967) has noted that 50 day old dark reared albino rats can perform successfully on the standard visual cliff apparatus within minutes of first visual experience, indicating an intact ability to discriminate gross depth difference. Tees (1974) has reported that dark reared Long -Evans strain rats do not improve on their ability to perform successfully on the visual cliff as rapidly as light reared controls after 20 days of age, while Walk and Walters (1973) have reported an impairment of fine depth discrimination after only 30 days dark rearing in the hooded rat.

TABLE 7.3 Summary of factors involved in patterned and diffuse light exposed and dark control rats, and their proposed importance on visual cortex acetylcholinesterase changes.

	AChE increased					Possible effect on V.C. AChE	Possible involvement of brain processes underlying visual learning
	L	CL	TL	DL	D		
Distal visual information present	Yes	Yes	Yes	Yes	No	-	-
Non-specific non-visual factors related to the first exposure situation	Yes	Yes	Yes	Yes	No	Nil	No
Proximal visual information present	Yes	Yes	Yes	No	No	Increased	Yes
Lens artefacts	No	Yes	Yes	Yes	No	Nil	No
Visual stimulation	Yes	Yes	Yes	No	No	Increased	No
Acquisition of visually mediated information	Yes	Yes	Reduced	No	No	Increased	Yes
Visually triggered cross-modal transfer effects	Yes	Yes	Reduced	No	No	Increased	Yes
Discrimination of colour and brightness differences	Yes	Yes	Reduced	No	No	Increased	Yes
Discrimination of object shapes	Yes	Yes	No	No	No	Other factors (also) involved	Yes
Gross depth discrimination	Yes	Yes	No	No	No	Other factors (also) involved	Yes
Fine depth discrimination	Reduced*	Reduced*	No	No	No	Other factors (also) involved	Yes
Acquisition of complex pattern discrimination and generalisation	Reduced*	Reduced*	No	No	No	Other factors (also) involved	Yes

* cf. Normals. Condition: L - patterned light exposed (no lenses). CL - patterned light exposed (with clear lenses). TL - diffuse light exposed (with lenses). DL - light exposed with opaque lenses. D - not light exposed.

Tees (1968) has found that 95 day old dark reared Long-Evans rats can discriminate simple geometrical figures, and lines in various orientations, as well as light reared controls, but that there was an impairment in acquisition of a complex pattern discrimination, and further (Tees, 1972) that the ability to generalise this visual discrimination was impaired. It is known that the albino rat can discriminate relatively well brightness (Walton and Bornemeier, 1939) and colour (Silver, 1967) differences, and no evidence has been put forward that these abilities are significantly impaired in the dark reared rats.

Opportunities for visually mediated new learning processes during initial exposure to light are certainly therefore present. Some aspects of the total visual stimulus can, on the results reported in this chapter, be excluded as being essential correlates of the increased acetylcholinesterase activity in the visual cortex following the visual experience. Although Chorover and Chase (1968) presented evidence that the albino rat can effectively improve visual pattern discrimination in spite of binocular occlusion, the type of occluder employed in the experiments described here contacts the entire corneal surface covering part of the scleral boundary,and would preclude such effects (Bonnet, 1973). As referred to earlier, Miller and Cooper (1974) report the failure of the normal rat to discriminate simple visually presented stimuli which were matched for brightness, following the application of translucent occluders. Their results also indicated that rats wearing translucent occluders can make discriminations on the basis of flux cues.

The conclusions of Table 7.3 cannot, however, be extended beyond the changes we have observed in acetylcholinesterase, even to the other cholinergic components. Changes in the levels of the degradative enzyme which is present in some non-cholinergic neurones (e.g. as is known in the cerebellum) may arise as a 'side effect' of other ongoing changes and may not be completely indicative of the organised cellular response to particular experience. This is substantiated from the results of two parallel studies on early learning in the chick (Rose, 1978b). Following exposure to an imprinting stimulus also involving visual stimulation, changes in acetylcholinesterase in forebrain areas are detected (Haywood, Hambley and Rose, 1975). Following a specific piece of new learning (passive avoidance of an aversive tasting bead), no changes in AChE are detected, whilst there is an increase of 21% in ^{3}H QNB binding in the forebrain which follows a specific time course (Rose, Gibbs and Hambley, 1979). On the other hand, the increases in AChE and ^{3}H QNB binding following visual experience in the rat follow a similar time course. No changes in either marker are detectable after the initial hour of exposure (unpublished observations), and both have returned to control levels after 24 hours of continuous exposure (Rose and Stewart, 1978). There is much evidence in the rat - and in other species studies - that memory for a learned behaviour has a short-term component lasting variously some minutes to hours, during which period consolidation can be fairly easily disrupted by electrical (Zornetzer and McGaugh, 1970) or pharmacological (Pearlman et al. 1961) intervention, presumably before brain processes underlying more permanent fixation are operative. An hypothesis which has been advanced (Rose and

Stewart, 1978) to account for the time course of changes in the cholinergic system following visual experience is that mobilisation of this system could represent an aspect of synaptic potentiation associated with the labile phase of the fixation of experience. The present results are consistent with and extend this hypothesis. An indication that an explanation in terms of simple light stimulation, which might be said, for example, to trigger biochemical changes in response to enhanced neuroelectric activity in the visual system is not entirely satisfactory, is perhaps provided by the results of Bigl and Schober (1977). These indicate that in the rat ACh is not likely to be involved in synaptic transmission of the visual system either at the cortical or subcortical level. It also appears that the majority of afferent cholinergic fibres to the rat neocortex may project from cells in the basal forebrain (Bigl and Biesold, 1978), an area which is known to be involved in central integrative processes involved in learning.

An explanation which may then be put forward and which, although speculative, can satisfactorily account for all the data, is that new learning phenomena occurring rapidly during the initial few hours of light exposure may require the involvement of central integrating processes in the basal forebrain projecting cholinergically to the neocortex. The enhanced synaptic facilitation thus occurring at the cortical level may be associated with the increases in acetylcholinesterase. This explanation is also consistent with the tendency which has been observed for increases in AChE to occur in the other cortical area studied, the increase in the anterior region being approximately one third (Wood and Rose, 1979b) to one half (Sinha and Rose, 1976; Wood and Rose, 1979a) that occurring in occipital

areas. Thus, although a concentration of the response is predominantly occurring in areas involved in reception and processing of visual information, this is not complete. It is reasonable to assume that if brain processes underlying learning and memory are altered following first visual experience, that other cortical areas including association cortex may display detectable neurochemical changes. Following the massive behavioural stimulus which initial visual experience represents, a diffuse activation of many fibre tracts may be involved, although not all changes may be currently detectable. The brain is primarily an organ of co-ordination and communication: with the environment through sensory systems and effectors; between parts of itself; and with other parts of the nervous system. The search for an increasingly small anatomical area in which to find specific biochemical changes to associate with an increasingly limited type of learning - which has been the prime focus of neurochemistry in this area - may yet end in the detection of a unique and massive change in a single brain fibre completely and only correlated with a specific piece of learned behaviour. Having achieved this, it may have completely explained nothing.

Conclusions

Using acetylcholinesterase as a biochemical marker, changes in visual cortex enzyme were monitored in groups of dark reared animals exposed to 3 h periods of controlled illumination. Levels in animals fitted with light diffusing scleral contact occluders, and therefore lacking highly structured visual information, were elevated 17% over dark control animals, but this was not different from the increase observed in animals exposed to normal lighting conditions. Control conditions indicated that the occluders had no effect on the enzyme levels measured, and that the increases

were specifically associated with certain aspects of the visual experience. The results were discussed in relation to behavioural evidence on visual abilities of animals in each of the light exposure conditions, and to evidence indicating the involvement of active cholinergic terminals in the rat cortex. They were interpreted as indicating that the functional stimulus for alterations in visual cortex acetylcholinesterase is liable to involve learning processes, but need not involve higher functions of perceptual processing.

CHAPTER EIGHT

FINAL DISCUSSION

The purpose of this final chapter is to summarize the main findings of this project and to relate these results to some of the conceptual issues raised in the Introduction. Some unanswered issues and suggestions for following these up are also indicated. The main themes of enquiry developed in this project may be encapsulated in the following questions:-

Is the extent and nature of the state dependent elements of cortical protein synthesis related to environmental and developmental variables?

Is the transient nature of the light induced increases in the levels of ACh markers related to a heightened neural activity associated with information acquisition or consolidation?

Are these changes independent and invariant, or do they interact with existing fluctuations in enzyme levels and/or behavioural measures?

Are the exposure related elevations in these markers dependent upon experimentally manipulated behavioural activity?

What visual stimuli are necessarily associated with an increased enzyme level during initial visual experience?

Some results bearing on these questions have been presented in a series of experiments as follows. Evidence emerged from some preliminary experiments described in Chapter 2 that the increase of incorporation of labelled lysine into visual cortex proteins associated with a 1 h exposure of 7 week old animals to light is differentially triggered dependent upon the environmental lighting/exposure conditions, the increase not being detected under conditions of very low ambient illumination. This result is consistent with previous findings from this laboratory (Rose, Sinha and Broomhead, 1973).

The large increase in the incorporation measure, but which was not visual cortex specific, which was detected on light exposure immediately after the age of natural eye opening, is indicative of a substantial and possibly functionally important change in brain metabolism at a significant developmental stage which interacts with the environmental stimulus. Experiments with another series of studies on visual experience in the rat, using a similar experimental design (Perry and Cronly-Dillon, 1978) have noted a large increase in the synthesis of a specific type of protein (tubulin) in the visual cortex at this age following eye opening and light exposure, but not eye opening alone.

The unexpected failure to detect an enhanced incorporation following light exposure of dark reared weanling animals was attributed to environmental factors rather than a true developmental difference, in view of subsequent results obtained with the acetylcholine system. These indicated a diurnal rhythmicity in the response to light exposure leading to a refinement of experimental design.

The cellular responses to dark rearing and subsequent light exposure involve a number of biochemical systems, and the demonstration of changed rates of incorporation of precursors into proteins reveals one aspect of this phenomena. Some evidence was presented in Chapter 3 of the possible functional and behavioural significance of changes in enzyme levels involved in ACh metabolism following visual experience. The increase in visual cortex acetylcholinesterase, following a three hour period of exposure, was found to be diurnally rhythmical, as were the endogenous levels of this enzyme in the motor cortex of dark reared and normal animals. The exposure-induced elevation, it was surmised, was related to the level of

visual attention during exposure. It was demonstrated that dark maintained animals displayed a circadian rhythm of behaviour both in constant darkness and interacting with time of light exposure, and this provided some indirect support for the above hypothesis. Chapter 6 presented results indicating that the light induced elevations in visual cortex AChE and mAChr protein were present following controlled manipulation of the behavioural activity level during light exposure, and further that this procedure resulted in measurable alterations in motor cortex biochemistry; these effects were detectable across a wide range of lengths of visual deprivation prior to new experience. It was not possible to relate these effects to the correlation which had previously been observed between the spontaneous levels of motor activity and the motor cortex enzyme levels in dark maintained animals, and the influence of stress effects were considered. An interesting differentiation of response between normally reared and dark maintained animals was observed in endogenous cortical AChE levels, and in the response to controlled locomotion. These effects were not investigated at different points of the circadian cycle of activity in these animals, and this might provide further useful information.

It was possible from the experiments described in Chapter 7 to resolve some questions concerning necessary visually present stimuli associated with the increase in visual cortex AChE, by manipulating visual input with corneal contacting lenses during light exposure. The results were interpreted in relation to the visual abilities of the animals under degrees of restriction of experience, and clearly indicated that a considerable attenuation of stimulus information did not

abolish this visual cortex biochemical response. There is no evidence to extend these conclusions beyond the enzymic marker which was studied.

The problems of interpreting experiments involving a change in the environment or behaviour of an animal even under defined experimental conditions, were alluded to in Chapter I, and will not be recapitulated here. The experiments described in this project have been one approach to interpreting the significance of some environmental variables associated with this experimental design. It cannot be said that the sum total of the evidence is sufficient to conclude that the transient changes in cholinergic synaptic markers are necessarily associated with learning processes which may occur on visual exposure. It is unlikely that the changes solely arise from an altered pattern of stimulated neuroelectric activity in geniculo-striate fibres releasing ACh, as all evidence indicates that this pathway is not mediated synaptically by this substance: nor would an explanation which admits of a diffuse cortical activation mediated perhaps by way of a cholinergic reticulo-cortical system account for the visual cortical specificity of the responses.

There were no significant differences in visual cortex enzyme activity levels in animals subjected to forced exercise, and hence presumably in an elevated state of behavioural arousal, compared to quiescent controls. The employment of the light controlling occluders enabled an experimental design where the only difference in experimental treatment between the exposed animals and controls was in relation to the light exposure, and it becomes consequently improbable that any other independent variables are of significance. Although unlikely it does not rule out, for example, an enhancement of fear level

associated with aspects of initial visual experience, or altered brain metabolism associated with an increase in blood flow specifically to the visual areas through sensory stimulation.

The majority of changes in visual cortex metabolism which have been observed consequent upon light exposure are of a transient nature, and are not detectable after 24 h of continuous exposure - with the exceptions of the activity of some lysosomal enzymes not showing a clear regional specificity (Sinha and Rose, 1976), and in particular the increase in a rapidly labelling and exported neuronal glycoprotein fraction (Rose and Sinha, 1974) which appears to be more permanent. This lability of biochemical modulation is in marked contrast to the permanent modification in cellular architecture following dark rearing and light exposure - the morphological correlates of which were referred to in Chapter I. It appears likely that some part of the biochemical changes will represent rapid homeostatic adjustments, perhaps associated with increased neuronal activity, rather than specifically reflecting a change in the pattern of neuronal connectivity.

Changes in cholinergic enzyme levels, as have previously been referred to, might represent an increase in the total number of enzyme molecules as one result of the enhanced synthesis of neuronal protein, or the activation of the preformed molecules. Although the turnover of acetylcholinesterase in rat cerebral cortex is quite rapid compared to most brain proteins, the half-life has been calculated as 2.84 ± 0.13 days (Wenthold et al., 1974). However, the isozymes of acetylcholinesterase have a wide range of half-lives, one isozyme (Davis and Agranoff, 1968) having been demonstrated to turn over with a period of about 3 h, and hence changes in the

total number of enzyme molecules in response to a three hour period of visual experience is a possible explanation.

Similarly, the half life of QNB binding protein appears relatively short (Rose, 1978a). The large increase in ^{3}H QNB binding to the muscarinic cholinergic receptor may indicate the formation of new cholinergic synapses or the facilitation of transmission at existing synapses, in response to a functional stimulus. The transient nature of this effect is interesting. It has been demonstrated that some neurones during normal development form transient synapses with turn-over rates of less than 21 h in the chick retina (Ruffolo et al., 1978). Conjecturally, the increase may represent an aspect of a response to initial visual experience which recapitulates a delayed ontogenetic maturation of 'fine tuning' synaptic specificity. As with the enzyme changes, biochemically this increase may represent new synthesis of receptor proteins. As has previously been referred to, this explanation is tentatively indicated, for the elevation in ^{3}H QNB binding is abolished if cerebral protein synthesis is simultaneously inhibited during light exposure (S.P.R. Rose, unpublished). This result is noteworthy in view of work on the kinetics of inhibition in the presence of puromycin, in the cultured chick muscle. This suggests that newly synthesised protein components of receptor molecules are present as a pool of precursor material which can supply the surface with new ACh receptors for several hours after inhibition of protein synthesis (Fambrough et al., 1978).

It is apparent that many of the behavioural issues surrounding learning, which were raised in Chapter 1, have not begun to be tackled either with this or other experimental approaches to neurochemical correlates of experience and learning. A description

of the types and temporal parameters of the molecular species involved in certain plastic adaptations to new afferent information, which may be taken as a model for adaptations underlying more specific forms of neuronal plasticity involved in learning, is perhaps an asymptotic limit to which this methodological approach may tend. The dynamic and modifiable brain-behavioural-environmental interaction is of such a complex nature that adequate translational languages to interpret even de facto correlative components are unsatisfactory. It is almost certain that many attempts to provide explanations of particular behavioural changes in terms of linear correlations with brain biochemical metabolism reflect anomalies of particular experimental designs. Nevertheless, considerable progress has been steadily achieved in this area, and at least the definition of objectives, which in some early studies were relatively unfocussed, have become clarified in the wake of experience, and some of the solutions have become clearer.

Finally, this project has left many questions unanswered and raised several new ones. With the experimental design employed, the effects of handling and of isolation contingent upon first exposure may not be equally applicable to each group - it might be objected, for instance, that the effect of social isolation may differentially stress the animals being concurrently visually exposed for the first time. Further controls could involve separation of animals into individual cages some time prior to experimental treatments, or alternatively splitting the dark reared animals into two groups some time prior to exposure - the experimental treatment simply involving removal of the cover from one box, or switching on

internal illumination in one box. This modification is not applicable to incorporation studies or other procedures necessarily involving handling of the animals, however.

A greater modification of experimental conditions could employ eyelid suturing and exposure at different ages during development of normal and dark reared animals, the closed eyes being sensitive to lighting changes but not to other visual information through the eyelids. It would be interesting to determine how prior exposure to diffuse light stimuli during development might affect the response to subsequent visual experience.

As have been discussed in Chapter 7, the visual abilities of dark reared rats appear to be largely unimpaired, although a direct demonstration of visual learning occurring during initial exposure and the extent of any cross-modal interaction could be instructive. This might involve the acquisition of a tactile object discrimination through association with negative reinforcement in darkness, followed by visual object presentation during light exposure. Behaviour towards the object could be recorded possibly in a forced choice discrimination with unexperienced objects, and responses compared with naive controls. This could be elaborated further by presentation of visible and apparently real but intangible solid objects - produced by polarised light in a shadow caster, or by projection through a fresnel lens - and measurement of startle responses.

Recording of eye movements could be employed as a further investigative tool during light exposure. Electrooculographic techniques could provide an indication of the amount and direction of eye movements in first exposed compared to normal and dark maintained animals, and it would be possible to

correlate these with visual presentation of stimuli in an otherwise blank visual field, giving a direct indication of visual attention to a variety of stimuli and its development through the exposure period. This could also be achieved with non-interventive techniques through recording of eye movements and other behavioural measures on film for subsequent analysis.

These further developments of behavioural methodology would go some way towards elucidating the undoubtedly complex processes which are initiated following the arrestment of and controlled exposure to visual experience.

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PUBLICATIONS

Some of the work described in this thesis has been presented at scientific meetings and has resulted in published communications and articles in learned journals, viz:

1. Wood, N. and Rose, S.P.R., (1978). Exposure of 7 week old visually naive rats to diffuse and structured light: effects on visual cortex acetylcholinesterase. Proc. Eur. Soc. Neurochem., 1, 565.
2. Wood, N. and Rose, S.P.R., (1978). Light exposure and non-spontaneous locomotor activity in visually deprived and normally reared rats: cortical effects in three components of the cholinergic system. Proc. Eur. Soc., Neurochem., 1, 566.
3. Wood, N. and Rose, S.P.R., (1979). Changes in acetylcholinesterase with light exposure, time of day and motor activity in the rat. Behav. and Neural Biol., 25, 79-89.
4. Wood, N. and Rose, S.P.R., (1979). The role of pattern vision in the increase of visual cortex acetylcholinesterase after exposure of dark maintained rats to the light. Neurosci. Lett., in press.